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Herpes Simplex Virus Type 2 Regulates the IFN-gamma Receptor Expression and Function

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Herpes Simplex Virus Type 2 Regulates the IFN-γ Receptor Expression and Function

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ABSTRACT

This study shows that HSV-2 can cause down-regulation in IFN-γR expression on the monocyte surface in both HSV-2 seropositive and seronegative patients. The objective of this work is to examine and explain the mechanisms involved in the viral down-regulation of IFN-γR. The first questions I tried to answer was whether humoral factors might participate in this down-regulation. Humoral factors were not involved in this phenomenon. Next, cell-to-cell interactions were examined. T, B or natural killer cell depletion experiments were conducted in peripheral blood mononuclear cells of both HSV2 seropositive and seronegative patients. The results suggest that NK cells and T cells but not B cells were involved in the IFN-γR down-regulation in HSV-2 seropositive patients. In addition, purified monocytes also demonstrated IFNγR down-regulation after HSV-2 exposure in seropositive patients. I concluded that, in HSV-2 seropositive patients, NK cells may have an inhibitory effect and T cells may have a facilitatory role in the down-regulation of IFN-γR. However, in HSV-2 seronegative patients, purified monocytes did not show an HSV-2-induced down-regulation of IFN-γR. Therefore, purified monocytes of seronegative patients need a cell-to-cell interaction to affect the IFN-γR expression. These results indicate a difference in the HSV-2 regulation of the IFN-γR between HSV-2 seropositive and seronegative patients.

The IFN-γ signaling pathway in monocytes from both HSV2 seropositive and negative patients was also investigated. The results suggest that this pathway is defective in HSV-2 seropositive patients in the presence of HSV-2. In contrast, there was no HSV-2 effect on the IFN-γ signaling pathway in seronegative patients. In conclusion, the HSV-2-induced down-regulation of IFN-γR may also affects the IFN-γ signaling in HSV-2 seropositive patients.

Since chlorpromazine inhibits receptor internalization by blocking clathrin-coated pits, this compound was used to determine whether IFN-γR down-regulation is mediated through this mechanism. Chlorpromazine has an inhibiting effect on IFN-γR down-regulation. Therefore, the internalization of receptors is the mechanism of IFN-γR down-regulation.

To sum up these results, it can be concluded that the HSV-2 have different ways to regulate IFN-γR receptors in seropositive and seronegative patients. This virus strategy may confer a survival advantage to the virus in patients with chronic HSV-2 disease.

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Abbreviations

Ab	antibody
AD	active disease
Ag	antigen
APC	antigen presenting cells
AS	asymptomatic
Bp	base pairs
CCAAT	binding sequence on DNA
CHX	cycloheximide
CMV	cytomegalovirus
CTL	cytotoxic T lymphocyte
CTL A-4	cytotoxic T lymphocyte antigen 4
°C	degree Celsius
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EBV	Ebstein Barr Virus
ECL	enhance chemiluminence
EDTA	ethylene-diamine-tetra-acetic acid disodium salt
ELISA	enzyme linked immunosorbant assay
Fc	receptor for Fc region of IG
FCS	Fetal Calf Serum
Gp	glycoprotein
HCMV	human cytomegalovirus
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)
HLA-DR	Human leukocyte antigen
HSV	Herpes simplex virus
HSV-1	Herpes simplex virus type 1
HSV-2	Herpes simplex virus type 2
HSV-7	Herpes simplex virus type 7
IFN	Interferon
IFN-γ	Interferon gamma
ICP(47)	viral TAP inhibitor protein
ISG	Interferon stimulated genes
ISGF	Interferon stimulated gene factor
IL	Interleukin
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-10	Interleukin-10
JAK	janus kinase
KDa	kilodaltons
LPS	Lipopolysaccharide from gram negative bacteria
M	Molar
MCF	mean channel fluorescence
ME	β-Mecaptoethanol

MHC	major histocompatibility complex
MCP	monocyte chemotatic protein
NAD	non active disease
Nef	HIV viral gene
NF	Nuclear factor
NK cell	natural killer cell
NO	nitric oxide
PBMC	peripheral blood mononuclear cells
PFU	Plaque forming unit
pg	picogram
SHP	tyrosine phosphatase
STAT-1	Signal transducers and activators of transcription
TAP	transporter associated with antigen processing
TCR	T cell receptor
Th2	T helper type 2 cell
TNF	tumor necrosis factor
Th	T helper cell
Th1	T helper type1
Th2	T helper type2
TWEAK	TNF-like weak inducer of apoptosis
UV	Ultra violet light
VZV	Varicilla Zoster Virus

INTRODUCTION

Epidemiology

Herpes simplex virus (HSV) exists in two forms that infect humans. Of the two strains, HSV-1 is associated with non-genital and genital infections, whereas HSV-2 is mainly associated with genital disease and neonatal infections. Genital HSV is a sexually transmitted infection (STI) with a seroprevalence of as high as 90% in some developing countries (1). In the United States (US), the seroprevalence of HSV-2 is around 25% but it has increased at a rate of 3% per year among women attending family planning clinics (1). Other US serologic studies demonstrate that the prevalence of HSV-2 antibodies is increasing (2). An increase in the prevalence of HSV-2 specific antibodies was also observed in Sweden, rising from 17 to 33 between 1969 and 1989 (3). Since the late 1970s, the prevalence of HSV-2 infection has increased by 30 percent and it is estimated that HSV-2 infection occurs in one of every five people in the world (4). There is consensus that the global prevalence of HSV-2 infections is increasing (2). Similarly, the number of cases of HSV-1 induced genital herpes increased significantly by 20% from 1978 to 1991(5). It is well documented that women have a greater risk of infection than men (5). In a recent study, susceptibility of mice to HSV-1 infection was gender-dependent, and IFN-γ played a more important role in protecting males than females (6). This may partially explain the differential susceptibility of the two genders to HSV infection.

General Architecture of Herpes viruses

The identification of Herpesviridae members is based on a unique architecture of the virion, which contains an icosadeltahedral capsid or core (100 nm in diameter) with a

linear double stranded DNA genome. It also contains a tegument, which occupies the space between capsid and envelope. On the outermost surface is an envelope, in which viral glycoproteins protrude from the surface.

Herpesvirus genomes may vary from virion to virion and the difference in size may be as high as 10 kbp and mostly contributed by terminal or internal reiterated sequences (7). The human herpesviruses can be classified into eight distinct types. Herpes simplex 1, herpes simplex 2, Epstein-Barr virus, cytomegalovirus, varicella-zoster virus, human herpes virus 6, human herpesvirus 7 and human herpesvirus 8.

Biological Properties of the Herpes Virus Family

There are several general properties shared by herpesviruses. First, all herpesviruses have a specific set of enzymes that participate in nucleic acid synthesis and metabolism (8). Second, viral replication takes place in the nucleus. Third, during the replicative cycle, the virus destroys the infected cell and the progeny viruses are released into the extracellular space. Fourth, the herpesviruses can remain latent in their hosts for a long period of time and only a small portion of viral genes are expressed. During this time, the viral genome is only partially expressed. It is believed that herpes simplex remains latent in cells of neuronal origin.

The Herpesviridae family contains three viral subfamilies, which include Alphaherpesvirinae, Betaherpesvirinae, and Gammaherpesvirinae (7). The members of the human alphaherpesvirinae include herpes simplex virus 1 and 2 and the varicella-zoster virus. The members of the betaherpesvirinae include cytomegalovirus, HHV6 and HHV7. The members of the gammaherpesvirinae include Epstein-Barr virus and HHV-8.

The members of alphaherpesvirinae have the following properties; they infect a broad range of their host's cell types, spread rapidly in culture and have a short reproductive cycle compared to betaherpesvirinae and gammaherpesvirinae (8).

The betaherpesvirinae have a restricted host range. They have a relatively long reproductive cycle and infection spreads slowly in tissue culture, through cell to cell transmission. The infected cells usually become enlarged (9). These viruses can establish latency in lymphoreticular cells and might even be found in this form in secretor glands, kidneys, and other tissues.

The gammaherpesvirinae host range is also limited (9). These viruses infect epithelioid cells, fibroblasts, T cells and B cells. Latent infection occurs in lymphoid tissue. EBV has two subtypes, A and B, which share a large homology and restriction endonuclease site conservation, but differ in their geographic distribution (10).

The HSV virion is 150 to 200 nm in diameter. It contains the 152-kbp, linear, double-stranded DNA. It is surrounded by the capsid, a 100 to 110 nm diameter protein shell with icosadeltahedral symmetry composed of 162 capsomeres, each with a hole along the long axis. DNA extracted from virions contains ribonucleotides, nicks, and gaps and has a G+C content of 68% for HSV-1 and 69% for HSV-2. The DNA consists of two covalently linked components containing unique sequences (U_L and U_s) that are bracketed by inverted repeats; 15kb of DNA sequence represent inverted repeats of terminal regions inserted between the U_L and U_s domains. Genes lie within both the unique and repeat sequences. More than 56 open reading frames (ORFs) have been discovered and HSV-1 is known to encode at least 84 proteins. Infected cells also contain transcripts from genome domains not known to encode proteins. These include the

latency-associated transcripts (LATs), which have been implicated in latency regulation, and an RNA (ORI_s RNA) derived by transcription of two of the three origins of viral DNA synthesis which map in the inverted repeats are present in two copies per viral genome. In productively infected cells, the HSV-1 replication cycle is 8 to 16h and HSV-2 replication cycle is even longer. HSV is a pathogen that initially infects the human body through the mucosal surface (11). The viral envelope glycoproteins attach to the cell membrane and assist in the viral penetration of the cell membrane initiating the virus replication cycle. HSV attaches to cellular receptors through glycoproteins gB, gC and gD in order to initiate viral penetration and infection. gC binds to the glycoaminoglycans (GAG), while gD binds to the Herpes Virus Entry (HVE) receptors (11a) (12). Glycoproteins gH and gL also participate in the fusion of the viral envelope to the cell membrane before cell penetration. After fusion of the HSV envelope with the plasma membrane, the viral capsid is released into the cytoplasm and then it travels to the nuclear pores where the viral DNA is released into the nucleus. RNA polymerase II transcribes viral DNA, generating different viral gene products that are required for viral replication. HSV genes and proteins are coordinately regulated and sequentially expressed in a cascade mode. The herpes simplex α genes/proteins are the first to be expressed. The maximal level of α protein expression occurs two to four hours after infection. There are five α proteins generated and they include ICP0, ICP4, ICP22, ICP27, and ICP47. All except ICP47 play a regulatory function, which is required for the synthesis of other protein groups. ICP47 may protect HSV-infected cells from cytotoxic T cell activity (9). The next cascade of events results in the synthesis of β proteins. Their synthesis levels peak five to seven hours post-infection. The β proteins include β1 (ICP8, the major DNA

binding protein) and β2 (thymidine kinase and DNA polymerase). The expression of β proteins is depended on the binding of an α proteins to the TAATGARAT promoter sequence. The γ genes are expressed last post-infection and are mainly comprised of structural proteins (8;9). Newly synthesized viral DNA is processed and packaged into preformed capsids in the nucleus. Processing involves amplification of some sequences and cleavage of viral DNA from concatemers that lack free ends. Seven viral proteins (UL6, UL15, UL17, UL25, UL28, UL32 and UL33) are responsible for cleavage-packaging events(13). Empty capsids are only scarcely enveloped, and those containing DNA fragments less than the genome length are generally retained in the nucleus (14). The virion envelope is required at the inner nuclear membrane and the maturation of envelope glycoproteins occurs as vesicles containing these virions move through the secretory pathway. Glycoprotein gK is involved in the translocation of infectious virions from the cytoplasm to the extracellular spaces (15). Also it was found that VP22 is responsible for intercellular HSV-1 transport via a nonclassical Golgi-independent mechanism (16).

Like other herpes viruses, HSV-2 has two special biological properties that influence disease. First, it can invade and replicate in the epithelium and in the central nervous system. Second, HSV-2 infects nerve endings and translocates to the nuclei of sensory ganglia by retrograde transport, where it establishes a latent infection that the immune system may not be able to detect.

Recurrences of HSV-2 are spontaneous and have been correlated with emotional or physical stress, fever, exposure to ultraviolet radiation (UV), tissue damage, and immune suppression (17). Latent virus can be reactivated and translocated either

unilaterally or bilaterally from trigeminal, sacral, and vagal ganglia of humans (17-19). However, the virus could be shed from its latent sites and into the mucosal surfaces without evidence of lesion formation. This phenomenon called asymptomatic viral shedding may explain the high prevalence of the infection in the general population. Many asymptomatic individuals could transmit the infection to their sexual partners without knowing that they are infectious. In these situations, it would be intuitive that asymptomatic HSV patients may have less frequent and less intense viral shedding than those who are symptomatic and have genital outbreaks of the disease. However, a study by Wald et al. (2000) showed that viral shedding in seropositive patients is frequent in the presence and absence of genital herpes-associated lesions (20). Little is known about the immune control, if any, of herpes recurrences or asymptomatic viral shedding.

Several studies have investigated the factors involved in viral latency and in the reactivation of genital herpes. The reactivation of herpes from latency may be regulated by viral factors or by the immune response to the viral infection. The latent associated transcript (LAT) is a viral factor that, as its name indicates, may regulate latency by an antisense mechanism (21). However, this proposed function remains controversial. Involvement of immune responses in the regulation of viral reactivation and latency remain controversial. One study suggested that the host immune response is not involved in HSV-1 latency (22). Contrary to these results, some investigations have shown that T cells regulate HSV latency and reactivation (23). Recent work by Lekstrom-Himes et al. (2000) examined the role of IFN-γ in HSV latency and reactivation (24). This group's findings suggested that IFN-γ had a preventive role in infection, partially suppressing ocular HSV infection in mice. IFN-γ had no effect on the regulation of viral latency or

reactivation. In addition, the herpesvirus load in infected cells could be affected by IFN-γ, but viral reactivation could still occur provided the threshold quantity of virus has been reached. In other murine model, IFN-γ had no effect on the neuroinvasiveness of HSV-1 (24). In the context of previously studied models of HSV infection, the functions of IFN-γ are to limit the quantity of virus during latency and to inhibit the spread of reactivated virus (24). The human translation of these models is supported by Torseth et al. (1986), who suggested that in contrast to individuals secreting low levels of IFN-γ, those patients who secrete higher levels of IFN-γ in response to HSV infection tend to have longer intervals between genital herpes recurrences (25). Clearly, more work needs to be done before the role of IFN-γ in the reactivation of HSV latency is understood. The work described in this thesis may increase our understanding of HSV pathogenesis.

Herpes Immune Response

The Immune Defense against a Viral Infection

Once the local non-specific barriers of infection have been broken and upon viral entry into the human body, there are two main stages of the immune response that may act to control the viral invader. The initial or innate immune response primes the more mature and delayed adaptive immune response. In the innate response there are three known major pathways that carry antiviral activity. First, the presence of the viral invader induces the production of interferons, IFN-α/β which induce activation of several antimicrobial effector mechanisms. These mechanisms include inhibition of viral protein synthesis, increase nitrogen oxide (NO) production by activating the nitrogen oxide synthetase (NOS), and activate cell-mediated lysis of infected cells by natural killer cells

(NK). Although IFN-α/β plays a central role in the innate immune response against viral infections, IFN-γ and TNF may have overlapping antiviral roles that were initially attributed to IFN-α/β. IFN-α/β, produced by leukocytes and fibroblasts, are quite different from IFN-γ. IFN-γ is produced by NK cell and effector T cells, thus IFN-γ is involved in both native and adaptive immunity and it appears later than IFN-α/β. IFN-γ facilitates the function of macrophage in adaptive immunity. On the other hand, IFN-α/β can facilitate the role of NK cell to kill infected cells in non specific innate immunity. IFN-γ can induce the NOS and TNF can synergize with other factors to induce NOS. Besides the NO antiviral defense, the humoral attack by the alternative pathway of the complement system may also play an essential role in the initial control of the viral infection by the innate immune response. The complement system can be activated after sensing viral elements in plasma during the initial round of viremia and can opsonize, destroy and limit the amount of virus load.

Monocytes and macrophages, NK cells, polymorphonuclear (PMN) cell, dendritic cells participate in the innate immune response. NK cells have antiviral effect by using 3 mechanisms. First of all, NK cells can be activated by IL-12 to produce IFN-γ. Secondly, IFN α/β can induce cytotoxic activity. The third NK antiviral function is when virus specific antibodies are induced, NK cells can use Fc receptors for immunoglobulins to lyse infected target cells through antibody-dependent mechanisms.

Similar to NK cells, macrophages have direct antiviral functions. Macrophages also produce a variety of cytokines, which include IL-12, TNF-α and IL-1α. Besides cytokine production Macrophages can also present the antigen to the adaptive immune system.

After 4-5 days, the effector lymphocytes start to dominate the antiviral function and it is a landmark of adaptive immunity commence. The antigens are carried to local lymphoid organs by the mobile antigen presenting cells (APC). The APC will present the antigen to naïve T cells. Then the naïve T cells will differentiate into armed effector T cells. The armed T cells will either stay in the lymphoid organ to activate antigen binding B cells or leave lymphoid organ to participate in the cell-mediated immune response in the infected area. The cytokines, secreted early in the innate immune response will determine which type of CD4 these cells will differentiate into. CD4 (Th-0) can either change into Th-1 or Th-2. The function of Th-1 cells is to activate macrophages and help them to destroy intracellular microorganisms more efficiently. The function of Th-2 cells is to activate B cells to differentiate and produce immunoglobulins, which provide a variety of effector molecules to the humoral immune response.

The B cell response assists in preventing re-infection and in controlling the spread of virus. IgA is an antimicrobial effector present at the mucosal surfaces. IgM is the dominant immunoglobulin in innate immunity and it switches to IgG during the adaptive immune defense. Some of the antibody class switching is T cell independent, and other responses require specific armed helper T cells. The IgM response is transient and the IgG response can persist for years. Upon primary infection, the antibody response becomes maximal in 2 weeks post infection within the spleen. Then the bone marrow becomes the main supplier of the plasma cells, which are the antibody factories of the adaptive immune response.

The Specific Immune Response to Herpes Simplex Infections

Natural Killer Cells

NK cells play an important role in the innate immune response against herpes infections. NK cells are able to sense, target and kill infected cells. This process is independent of antigen and is not limited by MHC restriction (26). NK lytic activity is "Signal Transducer and Activator of Transcription" (STAT-1)-dependent, but IFN-independent. The CCAAT/enhancer binding protein γ (C/EBPγ) is crucial for NK cell lytic activity and IFN-γ production (27). In NK cells with a defective IFN-STAT1 pathway, IL-12 can be used to partially recover cytotoxicity (27). Other cytokines, including IL-2, IL-15, and IL-18, enhance NK cell cytotoxicity. IL-15 is secreted by monocytes (28), along with IL-18, these two cytokines increases the expression of Fas ligand, while IL-18 enhance perforin/ granzyme expression, which mediate cell membrane damage of infected cells (29-31). IL-15 activates NK cells against herpesvirus infected cells, including HSV-1, Epstein-Barr virus, and human herpesvirus 6. This antiviral activity induced by IL-15 is dose, time and NK cell dependent (32). IL-15 also increases IFN-γ synthesis in both CD4+ T cells and NK cells. However, this effect is controversial and may require the presence of other cytokines. Lauwerys et al. (2000) and Fantuzzi et al. (1998) demonstrated that NK cells stimulated with IL-2 or IL-15 did not secrete IFN-γ, but NK cells stimulated with IL-12 or IL-18 did secrete this cytokine (33;34). Work by Ahmad et al. (2000) supports the argument that IL-15 derived from NK cells induces less IFN-γ than IL-12 derived from the same cells (28). Therefore, the ratio

of secretion of IL-12 to IL-15 can determine the amount of IFN-γ secretion, which in turn may influence the subsequent antiviral immune response in CD4 T-cells.

T cells

After infection, antigen presenting cells acquire HSV proteins and peptides, which are then presented, in a class II MHC context, to CD4 T cells. The MHC/peptide complex is recognized by the T-cell receptor in a highly selective and specific process. This recognition activates a cascade of events that result in the polyclonal expansion of T cells that are able to further proliferate in the presence of viral antigens during viral reactivation. HSV induce changes in both T-helper 1 and T-helper 2 immune responses. Th-1 responses include the secretion IFN-γ and IL-2 (35). Th2 responses include IL-4, IL-5 and IL-10 secretion (35). One report suggested that in HSV-1 reactivation, Th1 responses are inhibited, and Th2 responses are favored (36). However, there is evidence that IFN-γ plays a crucial role in inhibiting both HSV-1 and HSV-2 replication. In peritoneal and splenic macrophages of mice, IFN-γ has been shown to inhibit herpes replication, and encourages monocytes to lyse HSV-infected cells (37;38).

HSV-induces an increase of IL-10 and IFN-γ and a decrease in IL-4 secretion by T cells/PBMC from healthy individuals (35). This finding supports the view of a predominant Th-0 response with a bias towards a Th-1 response because it is well documented that IFN-γ secretion favors a Th-1 response. Milligan (1995) and (1997) used T-cell depleted mice to demonstrate that CD4+ T cells are vital in HSV infection (39;40). Since CD4+ T cells are the predominant source of IFN-γ, this explains why CD4+ T cells aid in controlling HSV-2 viral spread (41). There are other elements of the

adaptive immune response that are crucial for the control of HSV infection, such as CD8+ T cells that inhibit the spread of herpes infections even in the absence of IFN-γ. Holterman et al. (1999) determined that major histocompatibility complex (MHC) class I-restricted T cells play a crucial role in protection against neuroinvasive HSV infection in mice but largely depend on mechanisms other than IFN-γ (41). Furthermore, Tsunobuchi et al. (2000) demonstrated that CD8+ T cells secreting no IFN-γ-protected IL-2Rα-/- mice against HSV-2 infection (42). Therefore, the mechanism of CD8+ T cell defense may function independently on IFN-γ. Koelle et al. (2001) showed that upon initial HSV challenge, the viral tegument protein is one of the first target of HSV-specific CD8+ cytotoxic T-cells (43). In addition, CD4+ and CD8+ cytotoxic T-lymphocytes (CTL) may function in concert to fight the spread of HSV infection. CD4+ T-cells interact with antigen presenting cell (APC) to produce IL-2, which activates CD8+ MHC I CTL (44). It can be concluded that the CD4+ and CD8+ are both crucial to control HSV infection. IFN-γ is important for Th-1 CD4+ development in the HSV-specific antiviral response. In contrast, the antiviral effect of CD8+ T cells could be IFN-γ independent.

B cells

There is experimental evidence that B cells have an important role in the antiviral HSV response. Deshpande et al. (2000) demonstrated that B cell deficient mice were 100 to 1000 times more susceptible to HSV than normal mice (45). These mice lacked the ability to induce a humoral immune response against HSV as well as circulating IgM antibodies that serve as innate protection from herpes invasion. In humans, HSV-2

infection can induce both IgG and IgA antibodies specific to herpes, which limit the severity of infection (46).

Monocytes

Monocytes play an important role in protection against herpesvirus infections. These cells degrade proteins of the phagocytosed herpes virions (47). In addition, monocytes have the ability to provide innate resistance against HSV-2 before the onset of viral α protein expression (48). The cytotoxicity of monocytes towards infected cells is correlated with the production TNF-like weak inducer of apoptosis (TWEAK), which is a member of the tumor necrosis factor (TNF) family. TWEAK is able to induce apoptosis in HSV infected cells and in some cancer cells and it is expressed in monocytes in response to IFN-γ stimulation (49). Fresh human monocytes are non-permissive to HSV-2 infection because they have an innate resistance, inhibiting viral replication very quickly (48). The reason of non-permissiveness in monocytes is largely unknown, but it is believed that the induction of intracellular proteins is associated with this phenomenon (48). However, monocytes cultured for several days demonstrate a higher permissibility to HSV virus (50;51). This increased permissibility may be due to the differentiation of monocytes to macrophages, since these two cell types have different gene expression. Research by Tomura et al (1999) shows that the gene expression of chemokines such as IFN-inducible protein 10 (IP-10) and monocyte chemotactic protein-1 (MCP-1) are increased in macrophages (52). Mediated by augmented transcriptional activity, this increase is correlated with differentiation-dependent changes of the proteins bound to IFN stimulus response element (ISRE) and the kappa B site (κB), which is the

transcriptional binding site of nuclear factor kappa B (NF- κB) (52). Other work suggests that IFN-γ and TNF-α have roles in inflammation and immunomodulation implying that the synergism of IFN-γ and TNF-α can modulate chemokine gene expressions (53;54). Brunn et al (1999) showed that permissiveness is related to cytokine response in mononuclear phagocytes with HSV-1 infection (55). These cytokine includes IL-1, IL-6, TNF-α, which may maintain low HSV-1 infection susceptibility in monocyte (55). Although, there is an increase in chemokine expression in macrophages, the defect in some specific cytokine expression makes macrophage more susceptible to HSV-1 infection.

The interaction between CD40 and CD40 ligand (CD40L) in the mediation of chemokine production may be another potentially significant element of the immune response against HSV (56). CD40 belongs to the TNF receptor family, which is expressed on macrophages, B cells, activated NK cells, and dendritic cells (57;58).

Interferon-gamma mediated signal transduction

The IFN-mediated pathway depends on Jak1, Jak2 and STAT1 phosphorylation. Binding of the IFN-γ dimer to IFN-γ receptor 1 (IFN-γR1) triggers the activation of the receptor signaling complex (59). IFN-γ binds to IFN-γR1, then IFN-γR2 binds to IFN-γR1. IFN-γR1 associates mainly with Jak1, and IFN-γR2 associates mainly with Jak2. Upon oligomerization of the IFN-γ receptor chains, Jak1 and Jak2 kinases phosphorylate Tyr-457 of the IFN-γR1. The STAT1α SH2 domain interacts with the phosphorylated Tyr-457 of the IFN-γR1 chain and the Tyr residue of STAT1α is phosphorylated. This causes STAT1α form a STAT1 dimer, which then translocates to the nucleus and

interacts with the IFN-γ activation site (GAS). There are at least 100 IFN-associated genes expressed after IFN-γ stimulation, which in concert trigger part of the antiviral immune response (1;60;61). For example, HLA-DR expression increases with IFN-γ stimulation. Increase HLA-DR expression enhances antigen presentation to naïve T cells. IFN-γ can also increase the chemokine gene expression, such as IP-10, which plays an important role in the antiviral response.

As with many other biological processes, the IFN-γ/receptor signal transduction pathway is linked to other molecular events. This results in a tightly regulated environment. For instance, in the TNF-α signal transduction pathway, TNF-α causes dissociation of NF-κB from its inhibitor, IκB (62;63). The dissociated NF-κB travels to the nucleus and binds to a DNA sequence motif called the κB element (64). In a similar fashion, IFN-γ stimulated macrophages can induce DNA and NF-κB binding in endothelial cells (65). In contrast, IFN-α represses TNF-induced, NF-κB dependent, gene expression (66). Since the NF-κB plays a role in cell survival, the IFN-α induced repression of NF-κB can trigger cell apoptosis.

HSV and Evasion of the Immune Response

HSV has developed several strategies to suppress the immune system in order to survive. One such strategy is to inhibit antibody dependent cell lysis. The viral glycoproteins gE and gI bind to the Fc domain of IgG, inhibiting complement-enhanced antibody neutralization and antibody-dependent cellular cytotoxicity (67). Another evasive strategy was described by Favoreel et al. (2000), HSV inhibited CTL activity by infecting and destroying CTL (68). HSV could also eliminate antiviral T cells by

triggering apoptosis (69). Another method of evasion involves interfering with the MHC pathway in antigen presenting cells (APC) (70). As well, HSV can block MHC class I-peptide trafficking (68). Research by Mossman et al. (2001) has shown that the viral protein ICP47 can block host antigen presentation by preventing Transporters associated with Antigen Processing proteins (TAP) from transporting peptide to the endoplasmic reticulum (ER) (71). However, the immune system could also reverse some of these evasive mechanisms, for example IFN-γ can counteract the down-regulation of MHC I caused by HSV in human keratinocytes (72). This may be because IFN-γ increases the expression of TAP, and the limited amount of ICP47 cannot completely block the increased amount of TAP (68). The CD8+ T cell response may require IFN-γ from CD4+ T-cells to activate the MHC class I pathway in APC. Since the IFN-γ takes time to reverse the repression of TAP, it activates TAP in MHC I APC. This explains why CD8+ CTL response occurs two days after CD4+ T cell response (68). Furthermore, HSV may inhibit NK cell activity. When NK cells come in direct contact with HSV infected cells, the cytotoxic ability of the NK cells is compromised (73). In a study by York et al. (1993) HSV demonstrated its ability to transfer itself from cell to cell, including NK and lymphokine-activated killer (LAK) cells, which affected the effector functions of these cells (74).

Cytokine expression is also altered by herpes infection, and these changes may allow HSV-2 to evade immune detection (75). For instance, in HSV-1 infected human cells Transforming Growth Factor-β1 (TGF-β1) production is increased, perhaps contributing to immune system evasion (75); overexpression of TGF-β can suppress the development and differentiation of lymphocytes and monocytes/ macrophages (76;77).

HSV vaccine Candidates

The purpose of an HSV vaccine would be to prevent infection and HSV transmission, however, there has been little success in designing such a vaccine, till recently (67). Corey et al. (1999) devised a vaccine that included glycoprotein B2 and D2 (78), scheduled to be administered by intramuscular injection at the time of priming and 1 month and 6 months for the first and second booster doses, respectively. Clinical trials showed that both treated and control groups had similar rates of HSV infection. The ineffectiveness of such a vaccine could be due to the persistence of HSV in host cells without systemic viremia, making it impossible for antibodies to eradicate HSV from the host (67). A therapeutic gD2 DNA plasmid vaccine developed by Strasser et al. (2000) controlled the amount of virus passing in and out of nerve endings, although the vaccine did not improve virus clearance from infection sites (79). As well, the gD2 vaccine, which induced CD8+ T cells, did not significantly decrease recurrences in guinea pig models. CD8+ T cells might not be involved in the control of recurrent disease (79). Plasmid encoding chemokines have also been used as a vaccine component (80). Chemokines increase the expression of costimulatory molecules (B7-1 and B7-2), and also promote Th1 immune responses by increasing NK cell IFN-γ secretion.
More recently, a gD2 vaccine with a novel strong adjuvant containing 3-O-deacetylated-monophosphoryl lipid A and alum was able to protect a subgroup of HSV-seronegative women with an efficacy of 73%, but the vaccine did not protect men from infection (81). The explanation of the lack of efficacy in men is not known. However, the authors of this paper suggested that a biased Th1 response by women, especially interferon gamma secretion, might explain the enhanced protection in women by this vaccine.

Hypothesis

Human monocytes play a significant role in the immune response against HSV-2. This antiviral response is mediated mainly by interferon gamma secretion. The interferon gamma/receptor and signally pathway prepare these cells for an appropriate anti-HSV response. However, HSV-2 has developed strategies to evade the immune system. Viral evasion from the antiviral effect of IFN-γ may confer a survival advantage to this pathogen. HSV-2 may regulate the expression and function of the interferon gamma receptor on monocytes through an interferon-independent pathway.

Aims:

1. To characterize the effect of HSV-2 on IFNγR expression.
2. To explore the mechanism by which HSV-2 regulates the expression of the IFNγR on monocytes.
3. To assess the physiological impact of the IFNγR expression inhibition by HSV-2 on the IFNγ signaling pathway.

HSV antigen (Ag) effect on IFN-γR

Previous results by Singh et al demonstrated that HSV-2 seropositive patients with recurrent genital herpes had defective IFN-γ mediated immune response and dysregulated production of IFN-gamma (82). Asymptomatic HSV-2 seropositive individuals produced significantly higher levels of IFN-γ than individuals with recurrences or in the remission phase. In addition, monocytes from HSV-infected individuals did not exhibit IFN-γ-mediated regulation of the co-expression molecule B7 isoforms. Furthermore, monocytes

of individuals with genital herpes supported HSV replication compared to those of HSV-seronegative controls, and this enhanced replication was not inhibited by IFN-γ. These observations served as the basis to hypothesize that HSV-2 could modulate the expression of IFN-γR on monocyte surfaces. In addition, IFN-γR signal transduction pathway could be defective in patients with recurrent herpes, and normal in asymptomatic patients. Therefore, the first objective was to compare IFN-γR expression in asymptomatic, recurrent, and seronegative patients. Flow cytometry was used to monitor the expression of IFN-γ R in these patients. An *ex vivo* experiment with peripheral blood mononuclear cells (PBMC) incubated with inactivated HSV-2 (HSV-2 Ag) demonstrated that monocytes from HSV-2 seropositive patients have a larger down-regulation of IFN-γ R in response to HSV-2 Ag compared to seronegative patients (Figure1 a, b, c and Table 1). The HSV 2 regulation of IFN-γR was also examined in two *in vitro* models using a monocytic cell line and a neuronal cell, THP-1 and neuroblastoma cells. In contrast to the *ex vivo* experiments, the expression of IFN-γR did not change after exposure to HSV-2 Ag.

Differential IFN-γ secretions of asymptomatic, recurrent, and negative patients also confirmed previous results from this laboratory (82). The data demonstrate that asymptomatic patients have a higher level of IFN-γ secretion than recurrent patients. After HSV-2 antigen exposure, there is a high level of IFN-γ secretion in seropositive patients, whereas seronegative patients secrete a low level of IFN-γ. A high level of IFN-γ secretion may result in down-regulation of the IFN-γR, since IFN-γ induces internalization and use of receptors (83). However, a neutralizing antibody against IFN-γ did not obliterate the down-regulation of IFN-γR observed after HSV-2 exposure.

Similarly, the contribution of IL-10 to IFN-γR levels was also examined. Neutralization of IL-10 only had a partial inhibitory effect on the HSV-2 induced IFN-γR down-regulation.

The HSV envelope glycoproteins gD2, gB1, gC1 and gD1 were used to determine their effects on IFN-γR. The results suggest that all viral glycoproteins induced IFN-γR down-regulation, which was IFN-γ-dependent because an IFN-γ neutralizing antibody obliterated their effect on IFN-γR expression. The experimental data suggests that IFN-γR regulation by HSV-2 is both IFN-γ-dependent and IFN-γ-independent.

In order to further characterize the IFN-γR down-regulation in response to HSV-2, time course and dose response experiments were performed. The IFN-γR expression on monocytes was examined at different time intervals up to 48 hours after exposure to HSV-2 Ag. Maximal effects were observed at 24 hrs. All subsequent experiments were performed at this time interval. The dose response experiments demonstrated that a ratio of one cell to 1000 viral particles induced the greatest effect on IFN-γR inhibition. This cell: HSV-2 ratio was adopted in the rest of the experiments.

To determine whether HSV-2 regulation of the IFN-γR was mediated by the secretion of soluble factors from PBMC, the supernatant of the HSV-2 Ag stimulated PBMC was collected and incubated with the cell lines THP-1 and neuroblastoma. The HSV-2 Ag stimulated supernatant did not change IFN-γR expression, suggesting that a soluble factor was not involved in this phenomenon. To confirm this observation, a more direct method was used. A semi-permeable membrane was used to gauge the effect of soluble factors on IFN-γR. The secretion of soluble factors by PBMC (in response to HSV-2) did not induce the down-regulation of IFN-γR on purified monocytes.

Since the down-regulation of IFN-γR by HSV-2 was not mediated by the secretion of soluble factors, the down-regulation of IFN-γR in PBMC could be due to cell-cell interactions. To test this, individual cell types, such as T cells, B cells and NK cells were depleted from PBMC to observe the effect on IFN-γR down-regulation. Depletion of T cells, B cells and NK cells did not affect the down-regulation of IFN-γR induced by HSV-2. In addition, neutralizing antibodies and CTLA-4 were used to block CD28-CD80/86 interactions, and IL-2 secretion was examined. Results illustrated that IL-2 secretion was reduced as CTLA-4 was applied, while the HSV-2 induced down-regulation of IFN-γR was not affected. Finally, the expression of IFN-γR on purified monocytes was examined after HSV-2 Ag exposure. The purified monocytes demonstrated down-regulation of IFN-γR upon HSV-2 Ag exposure in seropositive but not in seronegative patients. These results suggested that the HSV-2 induced effect on the IFN-γ-independent IFN-γR expression is not likely due to soluble factors and that cell-cell interactions are not involved in this process. Most likely one or more HSV-2 antigens have a direct effect on monocytes to affect IFN-γR expression.

The next question was whether de novo protein synthesis was required for the regulation of IFN-γR by HSV-2. Fantuzzi et al. (2001) demonstrated that the inhibition of protein synthesis could affect the expression of chemokine receptors and cause the accumulation of chemokine receptor transcripts (84). As well, cellular response to the HIV glycoprotein (gp120) did not require de novo protein synthesis. Similarly, using cycloheximide (CHX) to inhibit de novo protein synthesis, I found that CHX had no effect on the HSV-2 induced IFN-γR down-regulation.

The effect of HSV-2 Ag on IFN-γR gene expression was also determined. Filter microarrays were employed to investigate the expression of genes for some chemokine receptors, cell surface receptors, and cytokines. The principle of filter microarray is based on the ability of P-32 labeled cDNA to bind to individual gene sequences spotted on a solid surface. More gene expression leads to more cDNA binding onto the filter array and results in a higher intensity spot. The cDNA sample exposed to HSV-2 Ag was detected and compared to control genes expressed on monocytes pre-incubated in media. Upon HSV-2 Ag exposure, differential changes in gene expression were examined in both positive and negative herpes patients. Gene expression on IFN-γR α chain did not change significantly, and gene expression on the β chain increased slightly in both seropositive and seronegative HSV patients. These results suggest that the inhibitory effects of HSV-2 on the IFN-γR expression are not regulated at the transcriptional level. The microarray result demonstrated that gene expression in chemokines and TNF family gene expression increased significantly after HSV-2 exposure.

The HSV-2 induced IFN-γR down-regulation may affect IFN-γ signaling, resulting in a defective antiviral response. As discussed in page 14, a decreased expression of IFN-γR could adversely affects the phosphorylation levels in the IFN-γR cytoplasmic region, resulting in decreased phosphorylation of STAT-1 and consequently decreased expression of interferon associated genes. Therefore, the extent of STAT-1 phosphorylation between HSV-2 Ag exposed monocytes and media cultured monocytes were compared, giving valuable information about the physiological state of the IFN-γ signaling pathway under the influence of HSV-2. STAT-1 phosphorylation was defective in monocytes of HSV-2 seropositive patients compared to seronegative patients, despite

higher STAT-1 levels in monocytes of seropositive patient than seronegative patient. These results suggest that HSV-2 could disrupt IFN-γ signaling by decreasing STAT-1 phosphorylation in patients with genital herpes.

The signaling pathway can also be examined by looking at the expression of Human Leukocyte Antigen (HLA)-DR molecules. In contrast to the STAT-1 experiments, HLA-DR was increased in monocytes after HSV-2 exposure in both seropositive and seronegative individuals.

Previous research indicates that thrombin plays a role in HSV-2 infection. According to a study by Pryzdial et al., CMV, HSV-1 and HSV-2 initiate thrombin production (85). We investigate the potential effect of thrombin on IFN-γR regulation.

In summary, this thesis examines potential mechanisms of IFN-γR regulation by HSV-2. Specifically, I looked at the receptor through the analysis of cell surface receptor expression, the contribution of soluble factors and cell to cell interactions, mRNA transcript levels, and signal pathways.

MATERIALS AND METHODS

Selection of patients in HSV-2 study

This study was approved by the Research Ethics Board of the Ottawa Hospital, University of Ottawa, Ottawa, Ontario. Patient history was recorded and PBMC were obtained with consent. Participants were divided into three groups: seropositive, recurrent patients with a history of genital lesions, seropositive, asymptomatic patients with no clinical history of genital lesions but serologic evidence of anti-HSV-2 antibodies by Western blot analysis, and seronegative patients who were healthy adult controls. For each groups, 5 patients were chosen to examine their IFN-γR expression.

Cells and virus

THP-1 (TIB 202), and neuroblastoma (IMR 32) cells obtained from the American Type Culture Collection (ATCC), were maintained in Iscove's Modified Dulbecco's Medium (IMDM; Sigma Chemical Company, St. Louis, Missouri) and supplemented with 10% fetal bovine serum (FBS) and 100u/ml of both penicillin and gentamicin.

Purified UV inactivated HSV-2 Ag and GD2 glycoprotein

Purified HSV-2 virions, strain G, were provided by Dr. Pryzdial (Canadian Blood Services, Ottawa, Ontario) and propagated in Vero cells (Advanced Biotechnologies, Columbia, MD). Virions were quantified and evaluated for purity by electron microscopy (86;87). Purification was performed by sucrose gradient binding as previously described in the published literature. HSV-2 was inactivated on ice for one hour at a distance of

5cm from a UV germicidal lamp (GE 030T8, 30W). Glycoprotein D2 was provided by Gary Cohen, University of Pennsylvania, Pennsylvania.

Non-purified heat inactivated HSV-2 Ag

Non-purified HSV-2 Ag was prepared inoculating human fetal lung cells (provided by the Division of Virology, CHEO) and the HSV-2 strain was purchased from ATCC. Briefly, 30 million cells were inoculated with $3x10^5$ plaque forming unit (PFU) of virus in 60 ml of media. Flasks were emptied, and 5 ml of media with 2% fetal calf serum (FCS) was added. $3x10^5$ PFU in 200µl was added and incubated at 37°C with 5% CO_2 for 2 hours. After adding 20ml of IMDM with 2% FCS, the flask was incubated overnight at 37°C with 5% CO_2. Plaque assays were performed at 24, 48, 72 hours post inoculation. Flasks were then subjected to three freeze/thaw cycles at -70°C and the sample was centrifuged for five minutes (min) at 2000 rpm. Supernatants were collected, and heat inactivated at 56°C for 2 hours. The mock-infected supernatant was prepared by a similar method but without the addition of HSV-2.

Isolation and culture of patient PBMC

Herpes negative adult peripheral blood was obtained from healthy volunteers. Herpes positive adult peripheral blood was provided by seropositive volunteers. All blood samples were collected in tubes containing sodium heparin (Becton Dickinson Vacutainer Systems, Franklin Lakes, New Jersey) after obtaining informed consent. PBMC was isolated by density gradient centrifugation using Ficoll-hypaque (Pharmacia, Baie D'Urf 130, Quebec), according to the manufacturer's instructions. Briefly, whole blood was

layered on top of Ficoll-hypaque in a ratio of 1:2, Ficoll-Hypaque to blood. Then the tubes were centrifuged at 20°C for 30 minutes at 1600 rpm. The PBMC layer was collected and washed three times with phosphate buffered saline (PBS). The PBMC was incubated in IMDM with 10% FCS and 5% CO_2 at 37°C. Dose response was performed with ratios of 1:1000, 1:100, 1:10, 1:1 and 10:1, cells to HSV-2 virion particles. Titrated HSV-2 virions were provided by Dr. E. Pryzdial at the Canadian Blood Agency in Ottawa.

Flow cytometric analysis

PBMC were collected from twenty-four well plates after 24 hours of culture. PBMC were washed with 0.1% sodium azide in PBS and put into flow cytometry tubes (Falcon, Lincoln Park, New Jersey).

The cells were then incubated with goat serum for 15 minutes to block non-specific antibody binding. PBMC were stained with flurochrome labeled monoclonal antibodies. Monocytes were stained with flouorescein isothiocyanate (FITC) conjugated antibodies to CD14 (Leu-M3, Becton Dickinson, Mississauga, Ontario). They were also labeled with R-Phycoerythrin (R-PE) conjugated mouse anti-human IFN-γR α chain (CD119) monoclonal antibody (BD Pharmingen International, Mississauga, Ontario). The cells were then incubated in the dark for 10 minutes at room temperature. PBMC were washed with PBS/0.1% azide. Data were acquired on a Becton Dickinson FACScan flow cytometer (Mountain View, CA, USA). When analyzing PBMC, the monocyte population was gated on the basis of $CD14^+$ cells. Data was analyzed using the WinMDI software package (J. Trotter, Scripps Institute, San Diego, CA).

IFN-γ and Neutralizing Antibody against IFN-γ and IL-10

Mouse monoclonal anti-human interferon-gamma neutralizing antibody (Biosource, Camarillo, California) was used to block the effects of secreted IFN-γ. Various amounts of the antibody were added in a preliminary experiment to determine the ideal neutralizing dose of this antibody, which was determined to be one microgram per milliliter. Two microliters of 0.5mg/ml IFN-γ neutralizing antibody were added to HSV-2 Ag, and after 24 hours incubation with PBMC, flow cytometry was performed to analyze surface IFN-γR expression. Enzyme Linked Immunosorbent Assay (ELISA) was also performed on cell supernatants to confirm the complete neutralization of IFN-γ. An anti-human IL-10 neutralizing antibody (R&D systems, Minneapolis, Minnesota) was used to block the effects of secreted IL-10. Four microgram of IL-10 neutralizing antibody in 1 μg/μl was added to the HSV-2 Ag. ELISA was performed to confirm complete neutralization. One hundred ng of recombinant human IFN-γ (Biosource, Camarillo, California) at 40ng/ml was also used to stimulate monocytes and THP-1 cells.

Negative selection of monocytes

Purified populations of monocytes were produced by negative selection using anti-CD2 (Pan T) and anti-CD19 (Pan B) antibody coated magnetic beads (Dynal, Lake Success, New York). Cells were mixed with beads at 4°C for one hour and resuspended in 2% FBS in PBS. Negative selection required beads in excess of ten times the number of T cells (40%) and B cells (20%) in the PBMC population. CD2 and CD19 attached with magnetic conjugated antibodies were separated using monocytes that did not attach the antibodies.

T cell, B cell, and NK cell depletion

T cells were depleted from PBMC by negative selection with anti-CD2 (Pan T) antibody coated magnetic beads (Dynal, Lake Success, New York). B cells were depleted from PBMC using negative selection with anti-CD19 (Pan B) antibody coated magnetic beads (Dynal, Lake Success, New York). PBMC were mixed with beads for one hour and resuspended in 2% FCS in PBS. Negative selection required beads in excess of ten times the number of T cells (represent 40% of PBMC population) and B cells (20%). NK cells were depleted from PBMC with anti-CD16 and anti-CD56 magnetic beads (Miltenyi Biotec, Germany). 10 μl each of anti-CD16 and anti-CD56 beads were added to PBMC and incubated at 4°C for 20 minutes. The magnetic column (MS column, Miltenyi Biotec, Germany) was primed with phosphate buffered saline (pH7.2) supplemented with 0.5% bovine serum albumin and 2 mM EDTA. PBMC with attached antibodies were then added to the primed column. The magnetic column bound NK cells and the remaining PBMC were collected. T cell, B cell or NK cell depleted PBMC were cultured with HSV-2 in a ratio of 1:1000 (cells to viral particles). Flow cytometry was performed as previously mentioned.

Measurement of cytokines

To examine the secretion of IFN-γ by HSV seropositive and seronegative patients, PBMC (1X 10^6/ml) were exposed to HSV-2 Ag (1:1000 ratio of cells to viral particles), as determined by dose response experiments in twenty-four well tissue culture plates (Falcon Labware, Lincoln Park, New Jersey). Supernatants were harvested after 24 hours

and frozen at -70°C. IL-2 concentration was examined before and after the addition of CTLA-4 fusion proteins (Research Diagnostics Inc., Flanders, New Jersey). ELISA plates (Nunc Immunomodules, Roskilde, Denmark) were coated overnight with PBS diluted, primary, monoclonal, antibodies against different various cytokines (IFN-γ AHC4432, 1μg/ml, Biosource, Carnarillo, California; IL-2 MAB 609, 4μg/ml, R&D Systems, Minneapolis, Minnesota). Plates were washed with PBS-Tween 20 (PBS-T) and blocked with a solution of 10% FBS of PBS in PBMC. The detection step used biotinylated monoclonal antibodies against each cytokine, diluted in a solution of 10% FBS of PBS (IFN-γ 0.5 μg/μl, AHC 4539, BioSource-Medicorp, Carnarillo, California; IL-2 50 μg/ml, BAF202, R&D Systems, Minneapolis, Minnesota). After washing, streptavidin-peroxidase (Jackson Immuno Research, West Grove, Pennsylvania) was added to 10% FBS of PBS to give a final dilution of 1:1000. Plates were washed again before the addition of the o-phenyenediamine substrate (OPD; Sigma, Oakville, Ontario), and the color formed by reaction was read at 450nm. Serially diluted recombinant cytokines were used as standards (IFN-γ, M-701, 500μg/vial, Endogen, Rockford, Illinois; IL-2, 202-IL, 1 μg/ml, R&D system, Minneapolis, Minnesota). Plate washes and readings were automated (Bio-Rad Laboratories, Hercules, California), and standard curves and concentrations were calculated using Microplate Manager 4.0 software (BIO-Rad Laboratories, Hercules, California).

Filter microarray

RNA was extracted from 10^7 T cells with RNeasy Kit (Qiagen, Mississauga, Ontario). 2 μg of RNA was mixed with 4 μl of human cytokine cDNA labeling primers

(Sigma Aldrich, Oakville, Ontario), and the volume was brought up to 15 µl with sterile distilled H_2O. The sample underwent annealing at 90°C for 2 minutes, followed by ramping at 42°C for 20 minutes. 333µM of dCTP, dGTP, and dTTP and 1.67 µM of dATP were added to 30 µCi dATP (α-^{32}P), 20 U ribonuclease inhibitor and 50 U AMV reverse transcriptase which constituted the master mix and was added to the annealed sample. The final reaction volume was increased to 30 µl with sterile dH_2O. The solution was mixed and incubated at 42 °C for 2-3 hours. Unincorporated radiolabeled nucleotide was removed from the labeled cDNA by Sephadex G-25 gel filtration spin column. The labeled cDNA probe was hybridized with the Panorama gene array (Sigma Aldrich, Oakville, Ontario). In the hybridization process, the arrays were rinsed twice with 50ml SSPE (0.18NaCl, 10mM sodium phosphate, pH7.7, 1mM EDTA) at room temperature for 5 minutes. Hybridization solution (with salmon testes DNA; Sigma-Genosys, PRHY0001, Oakville, Ontario) was heated to 65°C before use. The gene array was pre-hybridized using roller bottles at 65°C for1hr. Labeled cDNA generated was added to 2-3 ml hybridization solution in a 15ml conical screw-top tube. cDNA was denatured at 90-95°C for 10 minutes in a water bath. The hybridization solution was decanted from the pre-hybridized array and replaced with the denatured labeled cDNA. Arrays were hybridized for 12-18 hours at 65°C. Each array was washed for 2-3 minutes three times with 40-50 ml solution I (0.5x SSPE, 1% SDS), followed by two washes with the pre-warmed washing solution I in the hybridization oven at 65°C for 20 minutes. Arrays were then washed in 80-100ml of the pre-warmed solution II (0.1xSSPE, 1% sodium dodeyl sulphate (SDS) in the hybridization solution oven for 20 minutes. Each was then dried on blotting paper for 2-5 minutes, wrapped in plastic and exposed to a phosphor screen for 3

days. Phosphor screens were analyzed by image Quant (Molecular Dynamics) and Microsoft Excel.

Western blot analysis of JAK-STAT Pathway

160 million PBMC were collected from HSV seronegative or seropositive patients for Western blot analysis. The cells were incubated for 24 hours with media or HSV-2 in a ratio of 1:1000, PBMC to viral particle. Cells were then collected and sorted with magnetic beads as previously described. Each of the media and HSV-2 Ag pre-incubated samples was further divided into two parts, which were incubated in the presence and absence of recombinant, human IFN-γ (Biosource, Carnarillo, California) at a concentration of 200ng/ml for 20 minutes. Cells were lysed with lysis buffer (25ml 1M HEPES, 30ml 2.5M NaCl, 50ml 100% glycerol, 5ml 100% Triton X-100, 0.75ml 1M $MgCl_2$, 100ml 0.5M NaF, 1ml 0.5M EGTA pH 7.7, 288.25ml dH_2O, 250 μg/ml Aprotinin, 250 μg/ml Leupetin (Sigma, Oakville, Ontario), and 100μl of 1mM Sodium orthovanadate). Protein solubilization was conducted for 30 minutes on ice, followed by centrifugation at 14000g and 4°C for 20 minutes. Supernatants were collected, and the protein concentration was determined by Bio-RAD Protein Colorimetric Assay. Cell lysates were mixed with equal volumes of loading buffer containing 62.5mM Tris HCl (pH 6.8), 2 % SDS 25% glycerol, 0.01% Bromophenol blue, and 5% β-Mercaptoethanol (Sigma, Oakville, Ontario). Each well was loaded with 40 μg of protein, and boiled for 5 minutes before loading. Boiled samples were run in a 10% polyacrylamide gel containing 0.1% SDS. Proteins were then transferred to a Hybond C-extra nitrocellulose membrane (Amersham, Buckinghampshire, England) using a semi-dry transfer apparatus (BIO-Rad

Laboratories, Hercules, California) in a buffer containing 50mM Tris, 400mM glycine (BIO-Rad Laboratories, Hercules, California), 0.04% SDS, and 20% methanol. Membranes were blocked overnight at 4°C in PBS containing 0.1% Tween 20 and 5% non-fat milk then washed three times in PBS-T for 7 minutes in PBS-T. Washing was followed by incubation with primary anti-phospho-STAT1 polyclonal antibody (New England Biolabs, Mississauga, Ontario) at a concentration of 1 μg/ml in PBS-T containing 5% milk. Membranes were washed three times in PBS-T, followed by incubation with a polyclonal goat anti-rabbit antibody conjugated to horseradish peroxidase (Amersham, Buckinghamsphire, England) at a final dilution of 1:5000 in PBS-T containing 5% milk for 45min. The blots were washed three times in PBS-T, subjected to color development using ECL Western blotting detection reagents, and exposed to hyperfilm-Electro chemiluminence (ECL) (Kodaks, Rochester, New York). The membrane was stripped with buffer (62.5mM Tri-HCl pH 6.7, 100mM 2 ME, 2% SDS with 71μl DTT in every 100 ml of buffer) in water at 50°C H_2O for 30 minutes. The stripped membrane was then washed eight times with 5 minute incubations between each wash. The membrane was blocked with 5% skim milk in PBS-T, followed by incubation with primary anti-STAT1 polyclonal antibody (Santa Cruz Biotechnology Inc., Delaware, California) for 1.5 hour. The membrane was then washed three times with PBS-T and incubated with a polyclonal goat anti-rabbit antibody conjugated to horseradish peroxidase (Amersham, Buckinghamsphire, England) at a final dilution of 1:5000 in PBS-T containing 5% milk for 45min. Blots were washed three times in PBS-T, subjected to color development using ECL Western blotting detection reagents, and exposed to hyperfilm-ECL (Kodak, Rochester, NY).

Densitometry was performed with a GS-670 densitometer (BIO-Rad Laboratories, Hercules, California), and data were analyzed with the Molecular Analyst software package version 1.1 (BIO-Rad Laboratories, Hercules, California).

Inhibition of protein synthesis with cycloheximide (CHX)

PBMC were pre-incubated with 1 μg/ml and 2 μg/ml CHX (Sigma, Oakville, Ontario) for 30 minutes. The CHX incubation was followed by exposure to HSV-2 Ag for 24 hours. Cells were then subjected to analysis by flow cytometry as previously described.

Effect of soluble factor secretion on HSV-2 Ag regulation of IFN-γR

A semi-permeable membrane (Corning incorporated Life Sciences, Nagog Park, Massachusetts), with a 24mm diameter and 0.4 μm pore size was used to separate PBMC from populations of purified monocytes. The semi-permeable membrane was placed on top of a six well tissue culture plate (Falcon, Becton-Dickinson, Lincoln Park, New Jersey). PBMC cultured with HSV-2 Ag were placed on top of the membrane, while purified monocytes were cultured in a six well plate. The control consisted of sorted monocytes cultured with HSV-2 Ag in the absence of PBMC on top of the semi-permeable membrane of a six well plate was also used. Cells were then analyzed by flow cytometry as previously described.

Effect of CD28/B7 interactions on HSV-2 Ag regulation of IFN-γR

Different concentrations of recombinant human CTLA-4 (1, 2 and 4 μg/ml) muIgG fusion protein (Research diagnostics, Inc., Flanders, New Jersey) were used to block the CD28/B7 interaction. PBMC were incubated with HSV-2 Ag in the presence of CTLA-4 for 24 hours. IFN-γR expression was then examined by flow cytometry as previously described. Supernatants were collected, examined for IL-2 secretion, and analyzed by ELISA.

Culture with HSV glycoproteins

PBMC were cultured as previously described and incubated in the presence and absence of HSV-2 glycoproteins gB1, gC1, gD1, and gD2 (Gary Cohen, University of Pennsylvania., Pennsylvania). Ten μg of each glycoprotein were incubated with PBMC for 24 hrs. Following culture, PBMC was subjected to flow cytometry, as previously described.

Competition between the HSV-2 Ag and labeling antibody

PBMC were isolated as previously described and then fixed with 0.2% paraformaldehyde for 20minutes. The fixed cells were incubated with 5% goat serum for 15 min to block non-specific antibody binding. Fixed cells were exposed to different concentrations of UV inactivated HSV-2 Ag for 30min, excess HSV-2 Ag was washed off, and then stained with monoclonal IFN-γR antibodies to ensure that the interaction between the antibody and IFN-γR were not blocked by HSV-2 Ag. Concentrations of HSV-2 Ag used included 1:1000, 1:100, and 1:10, cells to viral particles. A negative

control, consisting of fixed cells without HSV-2 Ag, and a positive control, consisting of PBMC after 24 hours of HSV-2 Ag exposure, was included.

Chlorpromazine treatment

PBMC were cultured as previously described and incubated with HSV-2 Ag in the presence and absence of chlorpromazine (Sigma, Oakville, Ontario). Chlorpromazine was dissolved in DMSO and then it was put together with PBMC in two different final concentrations in the culture, 25 μM and 50 μM. Controls of these experiments were PBMC with DMSO with or without HSV-2 Ag incubation. Following culture, PBMC was subjected to flow cytometry, as previously described.

Statistical Analysis

Most data are expressed as mean channel fluorescence (MCF), which represents the intensity of fluorescent tags or labeled monoclonal antibodies binding to cell surface molecules. The MCF was measured by flow cytometry using standard parameters (82). The MCF in table 2 is the average of MCF plus minus standard deviation (SD). Human PBMC were exposed to media (control) or UV-inactivated HSV-2. The p value is calculated by using two tailed t-test comparing controls to experimental groups.

RESULTS

Aim1.- Characterization of the effect of HSV-2 on IFNγR expression.

HSV-2 regulates IFN-γ R expression in PBMC derived monocytes but not in THP-1 or neuroblastoma cells. The aim of these experiments was to investigate the role HSV-2 on IFN-γ R expression. Among different white cells, expression of IFN-γR on monocytes is important because monocytes play a central role in the IFN-γ-dependent antiviral immune response. These cells may present antigen and are able to recruit effector cells at the site of infection by releasing immune attractants (88). Flow cytometry was used to examine IFN-γR expression on monocytes from HSV-2 seropositive patients with recurrent disease. There were 5 patients in this group of patients. The same sample size was chosen with seropositive asymptomatic patients and seronegative patients. In order to specifically look at the IFN-γR on monocytes among many cell subtypes in PBMC, I stained not only the IFN-γR but also the CD14, which is a cell surface marker for these cells, since most of the CD14 expressing cells in PBMC are monocytes. Therefore flow cytometry is able to differentiate monocytes from other cells types in PBMC. PBMC cultures were incubated with heat inactivated HSV-2 Ag (at a 1:10 dilution) for 24 hrs. Mock-infected human fetal lung cell supernatant was used as control, which was heat inactivated and incubated with PBMC for 24hrs. The same experiments were performed with seropositive asymptomatic and seronegative patient samples. Since the results for each group fall into the same result range, I choose to present one out of 5 of the samples. The culture of PBMCs with HSV-2 Ag for 24 hrs demonstrated down-regulation of the expression of IFN-γR on monocytes from patients with seronegative and asymptomatic HSV-2 infections, as well as in HSV-2 recurrent individuals (Figures 1a, b, c). In

contrast, IFN-γR expression on both neuroblastoma and THP-1 cells was unaffected by HSV-2 Ag (Figures 3, 4). However, unlike monocytes in PBMC, THP-1 and neuroblastoma are cell lines, which are convenient in vitro experimental models formed by restricted number of cell subtypes. The reason to select THP-1 over other cell lines is that it has human monocytic cell properties. The human neuroblastoma cell line was selected because it is one of the few cell lines that has neuronal cell features, which is a cell that herpes simplex targets for latent infection and reactivation.

Flow Cytometry Assay Validation of experiments 1, 2, 3.

To validate this assay, several experiments were performed, including

1) Isotype control

Potential non-specific binding to IFN-γR by the tagged monoclonal antibodies, a non-specific murine antibody isotype control (antibody IgG2a), was investigated. The antibody isotope control IgG2a did not bind to the IFN-γR (Figure 4a).

2) Competitive binding assay between HSV-2 Ag and labeling Ab

To ensure that HSV-2 Ag was not binding to IFN-γR and blocking the epitope used by the anti-IFN-γR antibody, a competition assay was performed. Results indicate that increasing amounts of HSV-2 Ag do not decrease binding of the anti-IFN-γR antibody, and therefore do not block the ability of this antibody to bind IFN-γR (Figure 4b).

3) Intra and inter-assay variation

Intra-assay variation is a measure of the difference in results obtained from the same patient. This method was used to determine whether the observed effects were due to fluctuations in IFN-γR expression. PBMC from one patient was split into three equal

portions and stained separately before undergoing flow cytometry. The difference in MCF between the three portions is the inter-assay variation. Results demonstrate that the MCF of intra-assay variation is less than 0.5, and the MCF of inter-assay variation is approximately 1.0 (Figure4c). There may be some fluctuation due to samples being drawn at different times of the day, since IFN-γR expression changes slightly (less than 1.0) depending on the time of day that serum is drawn (Figure 4d).

Dose Response Curve of UV-inactivated and live HSV-2

In order to characterize the HSV-2 induced IFN-γ R response, a dose response curve was generated by varying the ratios of cells to viral particles of both live virus and UV inactivated HSV-2 Ag, using the following ratios: 1:1, 1:10, 1:100, 1:1000, and 10:1, with all dilutions done in culture media (Figure 5). One hundred virions per cell was the minimum number of viral particles required to engage cell receptors that recognize HSV-2 and then trigger IFN-γR down-regulation. Therefore, the dosage 1:1000 was adopted in subsequent experiments. Since live virus and UV-inactivated HSV-2 Ag behaved the same in dose-response experiments, UV-inactivated HSV-2 Ag was adopted for subsequent experiments.

Downregulation of IFN-γR by HSV-2 in different patient groups

Figure 1a
Negative

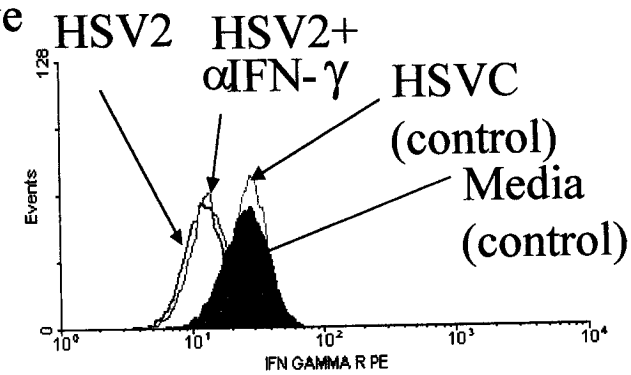


Figure 1b
Asymptomatic

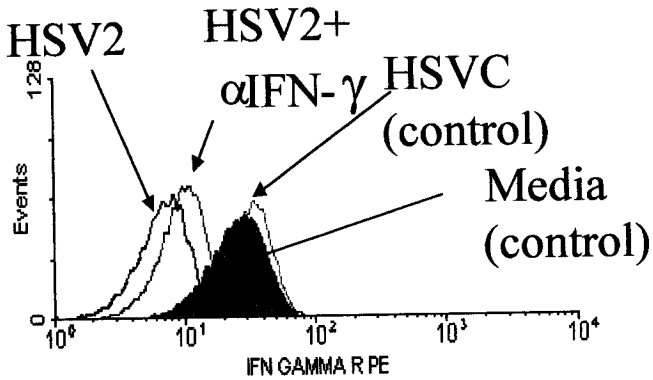


Figure 1c
Recurrent

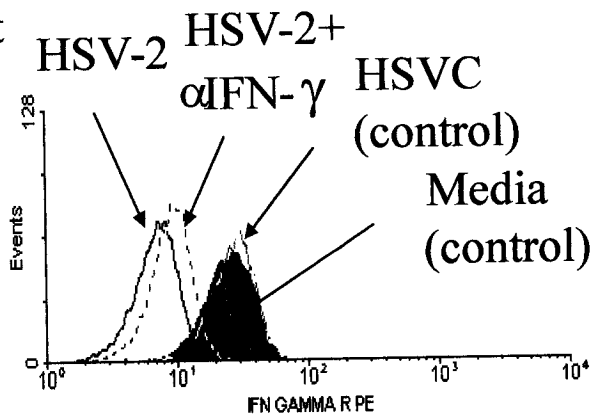


Figure 1 a, b, c

PBMC were labeled with CD14 FITC tagged Ab and IFN-γR-PE tagged Ab. Each subset demonstrates the flow cytometry analysis of PBMC expressed as mean channel fluorescence using both CD14 and IFN-γ R expression on monocytes. The peaks in the figures demonstrate CD14 positive cells with differing levels of IFN-γ R expression and according to the incubation conditions. PBMC were incubated in the presence of HSV-2 antigen alone (HSV-2), HSV-2 antigen and anti-IFN-γ (HSV-2+α-IFNγ), antigen control (HSVC) and media control.

Figure 1a:
Represents the monocytes of an HSV-2 seronegative volunteer.

Figure 1b:
Represents a patient with no history of genital herpes but who was HSV-2 seropositive by serology (Western Blot analysis).

Figure 1c:
Represents the experiments with monocytes of a patient with frequent genital herpes recurrences caused by HSV-2.

The IFN-γR expression in THP-1 and Neuroblastoma cell lines

Figure 2 : THP-1

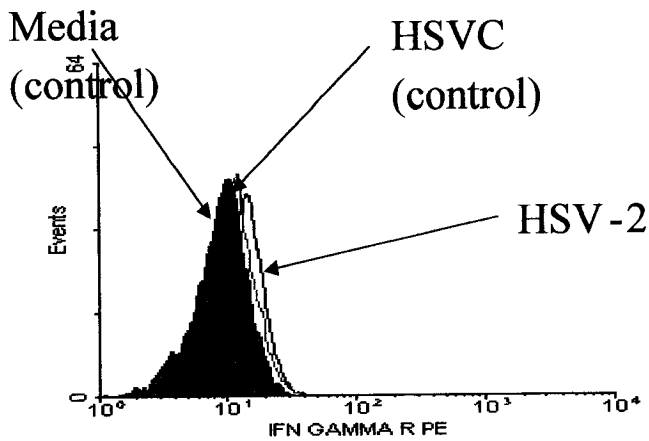
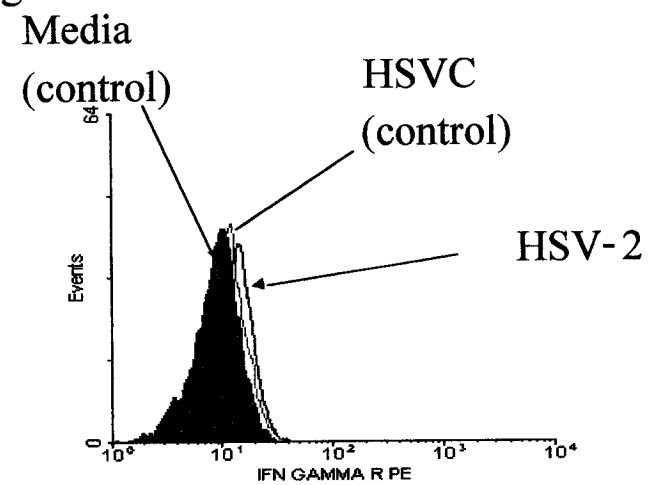


Figure 3 : Neuroblastoma



The figures 2 and 3 demonstrate the flow cytometry analysis of human THP-1 and neuroblastoma cell lines expressed as mean channel fluorescence using both FITC tagged CD14 and R-PE tagged IFN-γ R monoclonal antibodies. The peaks in the figures demonstrate CD14 positive cells with differing levels of IFN-γ R expression and according to the incubation conditions. Cells were incubated in the presence of HSV-2 antigen alone (HSV-2), mocked infected antigen control (HSVC) and media control.

Figure 2

Results: IFN-γR expression on THP-1 has no change after incubation with HSV-2 antigen.

Figure 3

IFN-γR expression in human neuroblastoma cells incubated in the presence of HSV-2.

Results: IFN-γR expression on the human neuroblastoma cell line has no change after incubation with HSV-2 antigen.

Figure 4a: The IFN-γR expression on THP1 cells, with IFN-γ(100ng) addition, inactivated HSV-2 Ag exposure

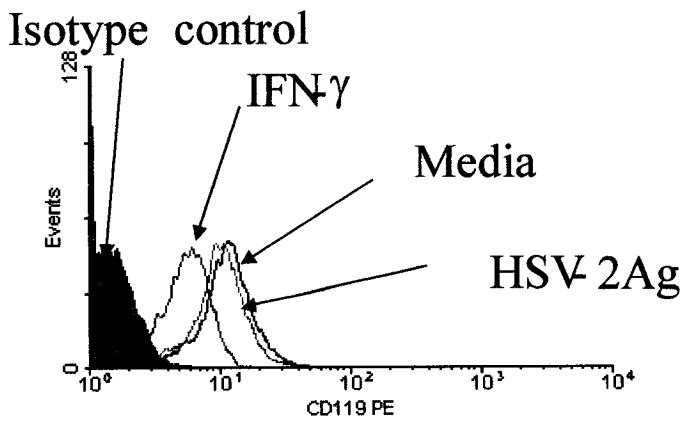


Figure 4b Competition assay between anti-IFN-γR Ab and HSV-2 Ag

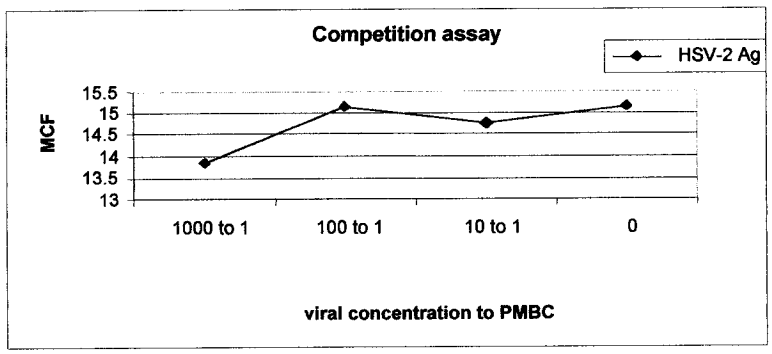


Figure 4a.

Objective: To show the control for potential non-specific binding to IFN-γR by the tagged monoclonal antibodies, a non-specific murine antibody isotype control (antibody IgG2a) was used. In addition, to demonstrate that the IFN-γ had the ability to down-regulate its receptor THP-1 was incubated with IFN-γ.

Results: The antibody isotope control IgG2a did not bind to the IFN-γR and IFN-γ down-regulated its own receptor.

Figure 4b:

Objective:
To ensure that HSV-2 Ag was not binding to IFN-γR and blocking the epitope used by the anti-IFN-γR antibody, a competition assay was performed.
Competition assay between concentrations of HSV-2 Ag and IFN-γR labeling antibody.

Results indicate that increasing amounts of HSV-2 Ag do not decrease binding of the anti-IFN-γR antibody, and therefore do not block the ability of this antibody to bind IFN-γR.

Figure 4c: Intra and inter-assay variation

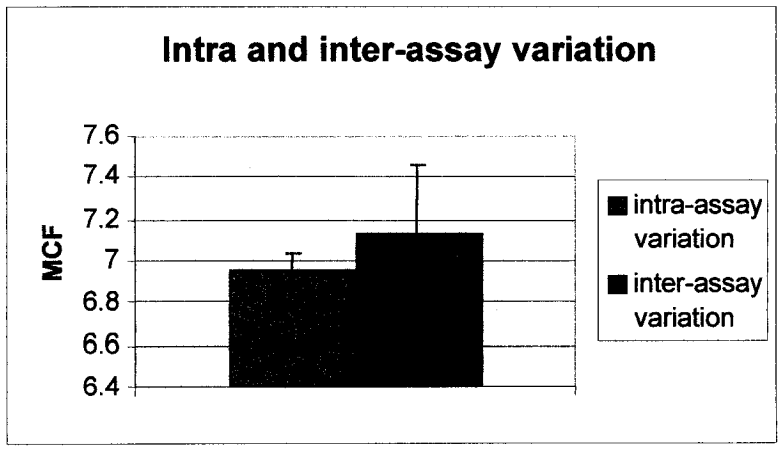


Figure 4d: Intraday variation of IFN-γ R

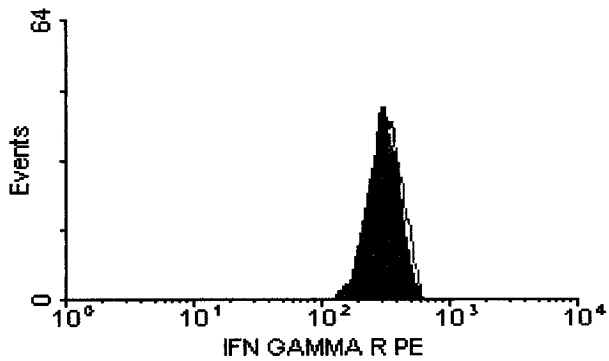


Figure 4c.

Objective:
To determine the intra-and inter-assay variation of stained PBMC samples measured as MCF.

Intra-assay variation is a measure of the difference in results obtained from the same patient. This method was used to determine whether the observed effects were due to fluctuations in IFN-γR expression. PBMC from one patient was split into three equal portions and stained separately before undergoing flow cytometry. The difference in MCF between the three portions is the inter-assay variation. Results demonstrate that the MCF of intra-assay variation is less than 0.5, and the MCF of inter-assay variation is approximately 1.0.

Figure 4d

Intra-day variation of IFN-γR expression in whole blood

Samples were drawn at different times of the day, IFN-γR expression changed slightly (less than 1.0) depending on the timing at which serum is drawn.

Figure 5. Virus: cell dose response curve of HSV-2 seropositivepatients

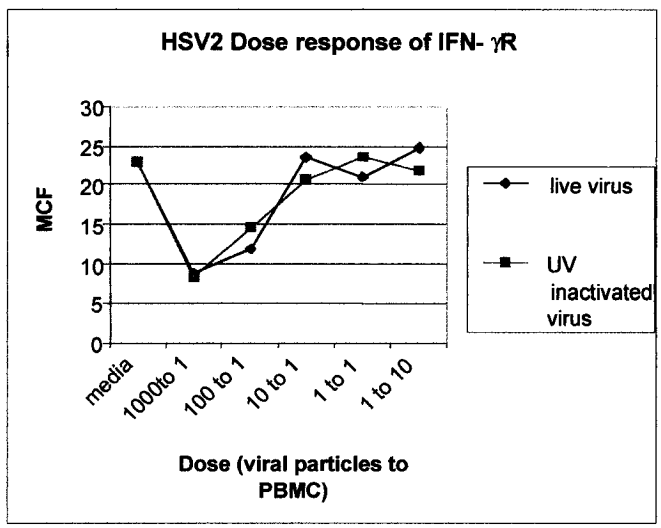


Figure 5.

The effect of UV-inactivated HSV-2 and live HSV-2 on the monocytic IFN-γR expression was examined after incubating varying amounts of PBMC and viral particle equivalents to generate a dose-response curve. PBMC from HSV-2 seropositive patients were incubated for 24 hrs with varing ratios of inactivated or live virus. The ratios of cells to HSV-2 virions were; 1:1, 1:10, 1:100, 1:1000, and 10:1. All dilutions were done in culture media. Cells were harvested, washed and double labeled with FITC tagged CD14 Ab and R-PE tagged IFN-γR Ab. These results represent 1 out of 3 experiments. Live HSV-2 and UV inactivated virions down-regulated IFN-γR in ratios of cells to virions of 1:1000 and 1:100 in the HSV-2-seropositive patient PBMC.

These results demonstrate that the HSV-2 antigen has different effects on IFN-γR expression in human monocytes, THP-1, and neuroblastoma cell lines. There was no regulation of the IFN-γR expression by HSV-2 in the cell lines. However, the observed IFN-γR down regulation by HSV-2 was similar in HSV-2 seropositive or seronegative individuals. In the neutralizing experiment, the anti-interferon neutralizing antibody was added at the same time as the inactivated HSV-2 Ag and incubated for 24 hrs. The negative control consisted of inactivated HSV-2 Ag and PBMC in culture for 24 hrs. An IFN-γ neutralizing antibody had little effect on the IFN-γR expression, which suggests that the IFN-γR down-regulation has an IFN-γ independent mechanism.

Time Course Experiment of the IFN-γR down-regulation by HSV-2

Time course experiments were done to measure and time the extent of IFN-γR down-regulation following exposure to HSV-2. PBMC were cultured with UV-inactivated HSV-2 Ag (at a ratio of 1000:1). The negative control consisted of PBMC cultured with supernatant of mock-infected cells (HSVC) or PBMC cultured with media only. After 0, 4, 8, 12, 24, 38, 48 hr incubation, IFN-γR, B7-1 and B7-2 expression were investigated. Monocytes from HSV-2 seropositive patients (n=3) showed that down-regulation of IFN-γR began after 6 hours of exposure to inactivated HSV-2 (Figure 6a). Down-regulation was most significant after 24 hours of viral exposure. In a time course experiment of an HSV-2-seronegative patient, IFN-γR down-regulation begins after 14 hours of exposure to HSV-2 and is maintained after 24hours (Figure 6b). After this, the degree of down-regulation decreases and IFN-γ R expression begins to increase after 40 hours of exposure (Figure 6b).

Previous work demonstrated that HSV-2 alters B7 expression on monocytes, which may affect the T cell response to HSV (82). However, it is still unknown how B7 regulation was altered by HSV-2 infection. B7 molecules interact with CD28 on the surface of T cells. This interaction may occur at the same time as the T cell receptor recognizes an antigen on the surface of APC such as monocyte/macrophage. B7/CD28 binding is necessary for the secretion of IL-2, resulting in a mature immune response against infection. To investigate whether the B7 molecules were co-regulated with the IFN-γR by HSV-2, expression of CD80 (B7-1) was also examined on monocytes during the time-course experiments. CD80 expression was co-regulated with the IFN-γR, increasing after 4 hours of exposure to HSV-2 (Figure 7a). Similarly, surface expression of CD86 (B7-2), another members of the B7 surface molecules, increased after 8 hours of incubation with HSV-2 (Figure 8a). Since IFN-γR was down-regulated with inactivated HSV-2 Ag, it would be interesting to know whether all the receptor or surface molecule expression would be decreased on monocytes. That is why B7-1 and B7-2 expression were investigated. However, the up-regulation of these molecules was only observed after HSV-2 infection. There was no effect from UV inactivated HSV-2 (7a and 8a, light blue line).

In the seronegative samples, CD80 and CD86 expression increase after 12 hours, and continue to increase after this time point (Figure 7b, 8b). These time course experiments suggest that HSV-2 down-regulates IFN-γR within 4 hours of exposure in HSV-2-seropositive patients. In contrast, IFN-γR down-regulation occurs within 15 hours of HSV-2 exposure in seronegative patients. In addition, the time course results demonstrated that the B7 molecules are also regulated in a similar time sequence as the

IFN-γR after HSV-2 antigen exposure but only in monocytes from HSV-2 seronegative individuals. Monocytes from HSV-2 seropositive patients did not up-regulate B7 molecules, demonstrating a defective response to inactivated HSV-2 and confirming previous observations by this group (82). Since the IFN-γR down-regulation is maximal at 24 hrs for both seropositive and seronegative patients, subsequent experiments were performed within that period.

Time-course IFN-γR Expression in HSV-2 seropositive and seronegative individuals

Fig 6a

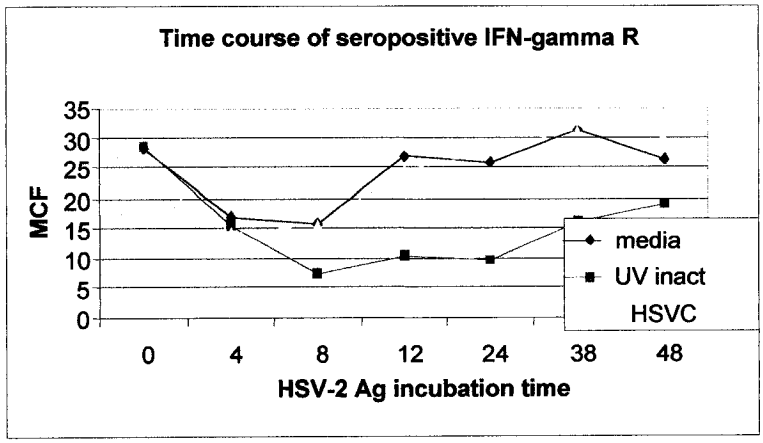
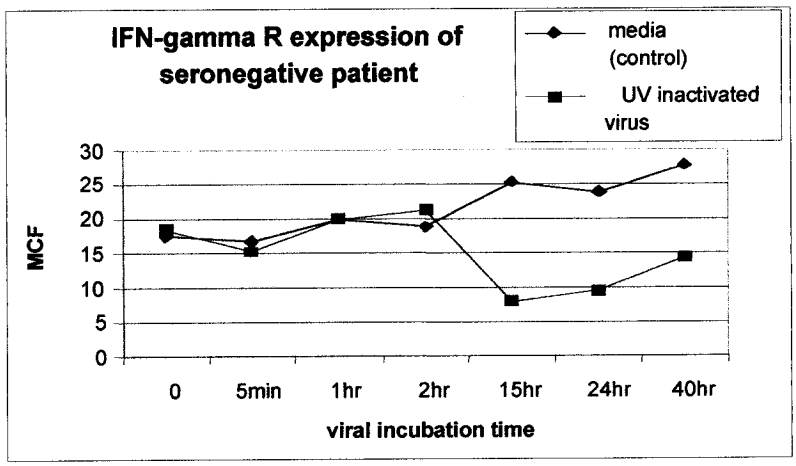


Fig 6b



IFN-γR down-regulation Time course in CD14+ cells

Figure 6a:
Seropositive patients:

Time course experiment to determine IFN-γR expression in PBMC monocytes from HSV-2 seropositive patients following HSV-2 Ag and HSVC (control) incubation.

PBMC were isolated and then cultured with live HSV-2 for 0, 4, 8, 12, 24, 38, 48 hrs at a ratio of 1000:1 (virions to cell). "UV-inact." virus represents the UV inactivated HSV-2 Ag culture with PBMC. HSVC represents supernatant of mock infected cells cultured with PBMC. Media control represents PBMC cultured with media only. At the end of each incubation period, PBMC were collected and labeled with FITC tagged CD14 and R-PE tagged IFN-γR. IFN-γR expression on monocyte was examined.

Figure 6b:
Seronegative patients

Time-course experiment to determine IFN-γR expression in PBMC monocytes from HSV-2 seronegative patients following exposure to UV inactivated HSV-2 Ag and control medium.

PBMC were isolated and then cultured with live HSV-2 for 0, 5min, 1, 2, 15, 24, 40 hrs at a ratio of 1000:1 (virions to cell). Media control represents PBMC cultured with media only. At the end of each incubation period, PBMC were collected and labeled with FITC tagged CD14 and R-PE tagged IFN-γR. IFN-γR expression on monocyte was examined.

Time-course CD80 Expression in HSV-2 seropositive and seronegative individuals

Fig 7a

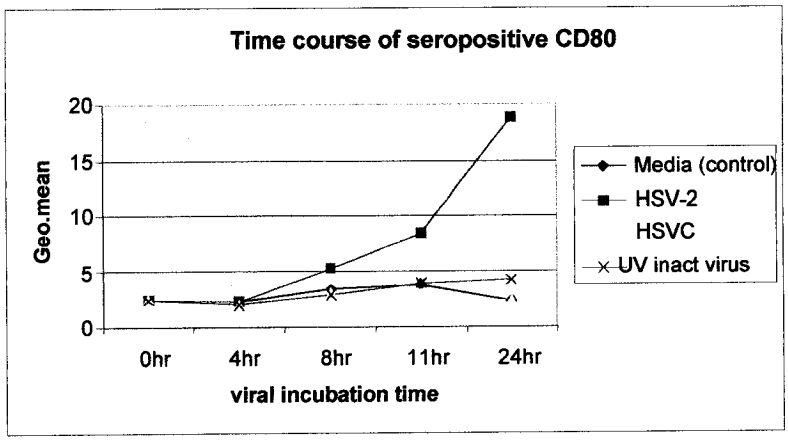
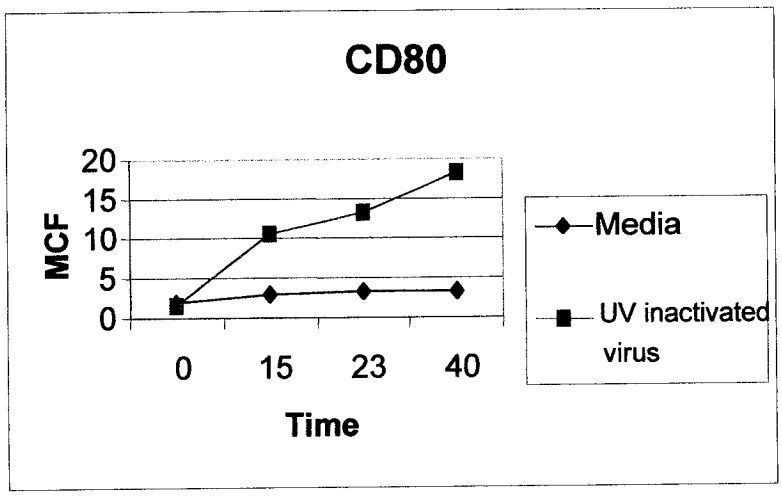


Fig 7b



Effect of HSV infection on CD80 expression in CD14-positive cells

Figure 7a:
Seropositive patients:

Time-course experiment to determine CD80 expression in PBMC monocytes from HSV-2 seropositive patients. PBMCs were incubated with intact HSV-2 (pink), inactivated HSV-2 (light blue) and HSVC (yellow) and media (dark blue).

PBMC were isolated and then cultured with live HSV-2 for 0, 4, 8, 11, 24 hrs at a ratio of 1000:1 (virions to cell), while media control represents PBMC cultured with media only. UV-inact. virus represents the UV inactivated HSV-2 Ag in ratio of 1000:1 and culture with PBMC. HSVC represents supernatant of mock infected cells cultured with PBMC. At the end of each incubation period, PBMC were collected and labeled with FITC tagged CD14 and R-PE tagged CD80. CD80 expression on monocyte was examined.

Figure 7b:

Seronegative patients:

Time-course experiment to determine CD80 (B7-1) expression in PBMC monocytes from HSV-2 seronegative patients following exposure to UV inactivated HSV-2 Ag (pink) and control medium (dark blue).

PBMC were isolated and then cultured with UV inactivated HSV-2 for 0, 15, 23, 40 hrs at a ratio of 1000:1 (virions to cell). UV-inact. virus represents the UV inactivated HSV-2 Ag. Media control represents PBMC cultured with media only. At the end of each incubation period, PBMC were collected and labeled with FITC tagged CD14 and R-PE tagged CD80. CD80 expression on monocyte was examined.

Time -course CD86 expression in seropositive and seronegative individuals

Fig 8a

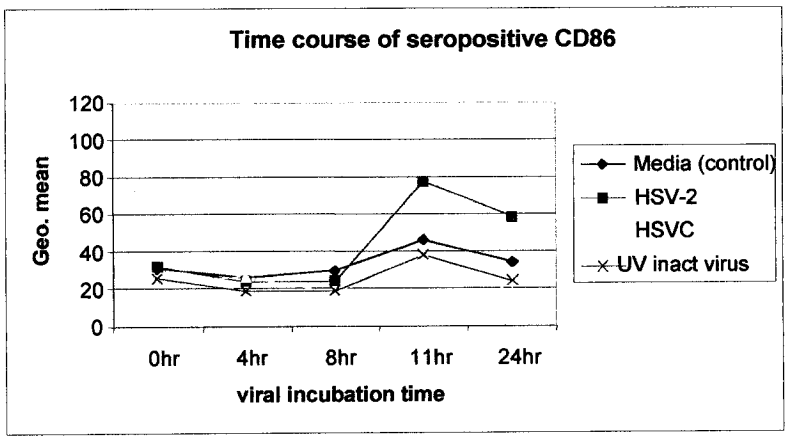
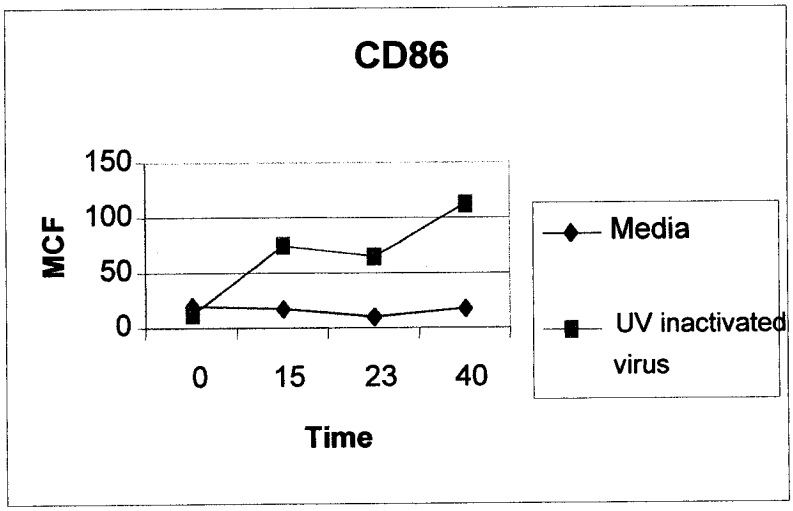


Fig 8b



Effect of HSV infection on CD86 expression in CD14-positive cells

Figure 8a:

Seropositive patients:

Time-course experiment to determine CD86 expression in PBMC monocytes from HSV-2 seropositive patients following live HSV-2, inactivated HSV-2, media and HSVC incubation. CD86 expression was measured after incubation with inactivated HSV-2 (light blue line), live HSV-2 (pink line), HSVC (yellow) and media (dark blue).

PBMC were isolated and then cultured with live HSV-2 for 0, 4, 8, 11, 24 hrs at a ratio of 1000:1 (virions to cell), media control represents PBMC cultured with media only. UV-inact virus represents the UV inactivated HSV-2 Ag in ratio of 1000:1 and culture with PBMC. HSVC represents supernatant of mock infected cells cultured with PBMC. At the end of each incubation period, PBMC were collected and labeled with FITC tagged CD14 and R-PE tagged CD86. CD86 expression on monocyte was examined.

Figure 8b:

Seronegative patients:

Time-course experiment to determine CD86 (B7-2) expression in PBMC monocytes from HSV-2 seronegative individual following exposure to UV inactivated HSV-2 Ag and control medium.

PBMC were isolated and then cultured with UV inactivated HSV-2 for 0, 15, 23, 40 hrs at a ratio of 1000:1 (virions to cell). UV-inact virus represents the UV inactivated HSV-2 Ag. Media control represents PBMC cultured with media only. At the end of each incubation period, PBMC were collected and labeled with FITC tagged CD14 and R-PE tagged CD86. CD86 expression on monocyte was examined.

Aim 2.- To explore the mechanism by which HSV-2 regulates the expression of the IFNγR on monocytes.

Comparison of IFN-γR expression in Monocytes after culturing PBMC with Purified Viral Glycoproteins or UV-Inactivated HSV-2 Ag.

GD2 is a glycoprotein essential for HSV-2 cell attachment and entry (89). Due to the importance of GD2 in viral infection and the effects of HSV-2 described above, I investigated the effect of this protein on IFN-γR expression. GD2 at a concentration of 5μg/ml was cultured with PBMC for 24 hrs. After incubation, PBMC were labeled with both R-PE tagged IFN-γR Ab and FITC-CD14 Ab. Each group had three individuals (n=3) and the results in each group were in a similar data range. Therefore, I have chosen one individual from each group to represent the results.

GD2 decreased IFN-γR expression on monocytes from both patients with recurrent genital herpes and asymptomatic HSV-2 seropositive patients (Figure 9a, c), but it did not have an effect on monocytes of HSV-2 seronegative individuals (Figure 9b).

In previous published results, cells from asymptomatic, recurrent, and negative patients produced different levels of IFN-γ in HSV-2 infection (82). The data demonstrate that asymptomatic patients have a higher level of IFN-γ secretion than recurrent patients. After HSV-2 antigen exposure, there is a high level of IFN-γ secretion in seropositive patients, whereas seronegative patients secrete a low level or no IFN-γ. It is interesting to know whether a high IFN-γ secretion is correlated with a large down-regulation of IFN-γR, since there is some evidence to suggest that IFN-γ can induce internalization of its own receptors (83).

Downregulation of IFN-γR

by GD2 is IFN-γ dependent

Figure 9a

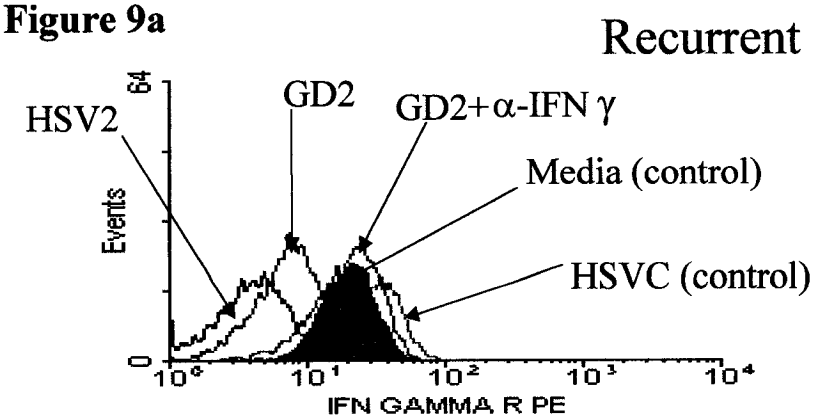


Figure 9b

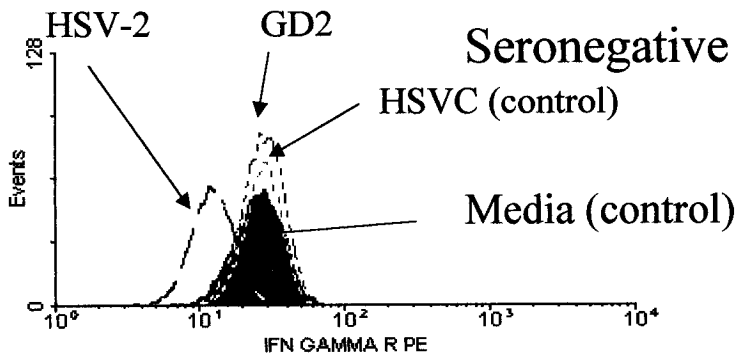


Figure 9c

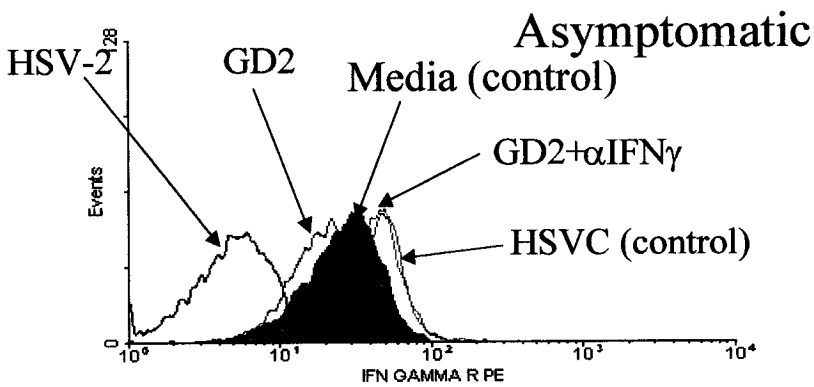


Figure 9a, b, c.

Objective: To determine the effect of glycoprotein D2 on IFN-γR expression in monocytes from HSV-2 seropositive patients. The experimental conditions of 3a, 3b, 3c was similar to that of 1a to 1c. Anti-IFN-γ was added to neutralize the activity of this molecule and to examine the dependence of the IFN-γR down-regulation on IFN-γ.

PBMCs were incubated with HSV-2, GD2, GD2+αIFN-γ, HSVC, and medium (control). a) Samples from an HSV-2 seropositive recurrent patient, b) HSV-2 seronegative, and c) HSV-2 seropositive asymptomatic patient.

Table 1. Summary of figure 1, 9, 10, 11

EXP#	Status	Media	HSV-2	HSVC	HSV2+ αIFN-γ	GC1	GD1	GD2	GD2+ αIFN-γ	HSV2+4 ul
		Control								anti-IL10
1	AD	24.74	6.76	26.72	8.9	20.47	15.89	9.91		
2	AS	25.05	6.65	30.43	9.54	10.73	10.66	20.97		
3	AS	24.9	4.93	39.41	N/A	N/A	N/A	19.5	38.95	4.94
4	AD	19.68	3.93	28.7	N/A	N/A	N/A	7.06	19.12	6.85
5	Neg	25.12	12.06	27.13	13.66	N/A	N/A	N/A	N/A	9.27

Table 1 is the summary of figure 1 (a, b, c), figure 9(a, b, c), figure 10 and figure 11. It provides the relative values of IFN-γR expression and the effect of anti interferon gamma neutralizing antibody, anti IL-10, glycoproteins GC1, GD1, GD2, and the combination between anti interferon gamma with GD2 on the IFN-γR expression. Monocytic IFNγR expression is measured as the intensity of the mean channel fluorescence (MCF) by flow cytometry. PBMC were incubated with HSV-2, HSV-2+αIFN-γ, HSVC, GC1, GD2, GD2+αIFN-γ, and media for 24 hrs. Fluorescent antibodies were then reacted with the cells to identify $CD14^+$ cells and the level of IFN-γR expression. AD represents patients with genital herpes with frequent recurrences, AS represents HSV-2 seropositive asymptomatic patients and Neg represents HSV-2 seronegative patients.

When neutralizing IFN-γ antibody [α-IFN-γ (Ab)] was added to the GD2 incubated sample, the down-regulation of IFN-γR expression was completely obliterated in both asymptomatic and recurrent HSV-2 seropositive patients (Figure 9a, c). The flow cytometry mean channel fluorescence (MCF) of IFN-γR expression on monocytes cultured with GD2 alone and GD2+αIFN-γ in both asymptomatic and recurrent patients is summarized in Table 1. In asymptomatic patients, MCF decreased from 24.9 to 19.5 upon GD2 exposure and returned to 38.95 in the presence of neutralizing anti-IFN-γ antibody (Table 1; experiment 3). Similarly, in a patient with recurrent genital herpes, the MCF decreased from 19.68 to 7.06 upon GD2 exposure, returning to 19.12 in the presence of neutralizing anti-IFN-γ antibody (Table1; experiment 4). In contrast, GD2 did not induce any IFN-γR down-regulation on monocytes from PBMC of seronegative patients (table 1; experiment 5). IFN-γ secretion was detected by ELISA in both HSV-2 asymptomatic and recurrent patients, but was undetectable in HSV-2 seronegative patients. In addition, the UV-inactivated HSV-2 Ag induced more IFN-γR inhibition than GD2 in both HSV-2 seropositive patients. Since the down-regulation of IFN-γR expression by GD2 can be inhibited by neutralizing anti-IFN-γ antibodies, this down-regulation is IFN-γ dependent.

GB1, GC1, GD1 are glycoproteins expressed on the surface of HSV-1. The reason these glycoproteins were used instead of the HSV-2 glycoproteins is that they were available to us through collaboration with the University of Pennsylvania Herpes Research Group. These HSV-1 proteins have 50 to 80% homology to the HSV-2 counterparts, which were not available. The effect of these glycoproteins on IFN-γ secretion and IFN-γR expression was examined. IFN-γR expression was similarly down regulated and IFN-γ

secretion increased by each glycoprotein in cells from seropositive patient, suggesting that HSV-1 glycoproteins play a role in regulation of IFN-γR expression on monocytes (Figure 10).

The Glycoprotein GB1, GC1 and GD1 downregulate IFN-γR

Figure 10

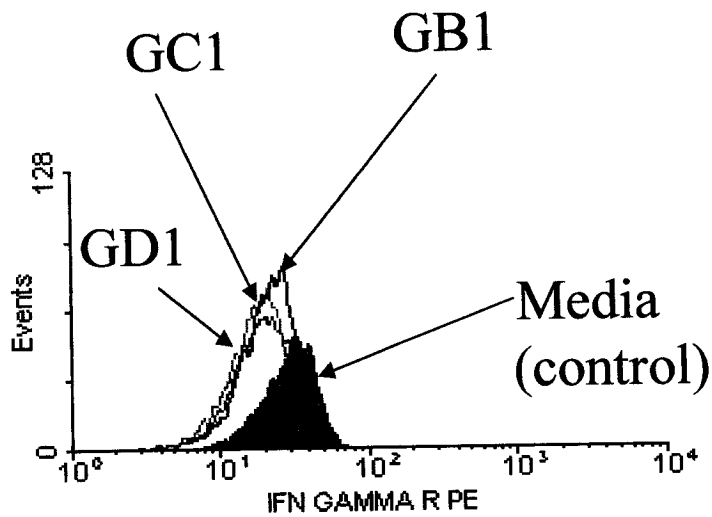


Figure 10:

Objective: To determine the effect of glycoprotein GB1, GC1 and GD1 from HSV-1 on IFN-γR expression on monocytes from HSV-1 and HSV-2 seropositive patient. The experimental conditions in this experiment were similar to that of 1a to 1c. GB1, GC1, GD1, and media were incubated with PBMC to determine monocyte IFN-γR expression by flow cytometry as explained in material and methods.

Result: The results are similar to the GD2 experiment. IFN-γR expression was down regulated in response to each glycoprotein, suggesting that HSV-1 glycoproteins also play a role in regulation of IFN-γR expression on monocytes.

Effect of IL-10 on IFN-γR downregulation

Figure 11

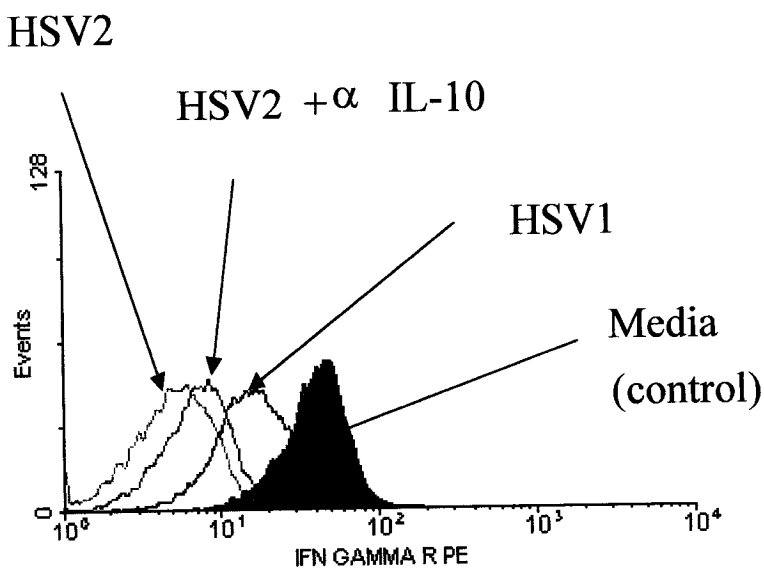


Figure 11.

Objective: IL-10 is believed to be an IFN-γ antagonist; therefore, the effect of anti-IL-10 neutralizing antibody was examined on HSV-2 seropositive and seronegative patients.

IFN-γR expression in monocytes after incubation of PBMC and HSV-1, HSV-2, HSV-2+αIL-10, and medium. PBMC were collected from HSV-2 seropositive, asymptomatic patients.

Results: These results suggest that IL-10 is not responsible or has little effect on the IFN-γR down-regulation induced by HSV-2 antigens.

Effect of HSV-2 Ag on the expression of IFN-γR on monocytes of HSV-2 seropositive patients and seronegative volunteers

HSV-2 Ag can down-regulate IFN-γR on monocytes from HSV-2 seropositive and seronegative patients. These results are expressed as mean channel fluorescence, so the relative changes under different experimental conditions can be compared. In experiment 1 (Table 1), monocytes in the patient with recurrent genital herpes experienced down-regulation of IFN-γ R with the MCF dropping from 24.74 to 6.76. After the addition of neutralizing anti-IFN-γ antibodies, IFN-γR expression increased slightly from 6.76 to 8.9. Similarly, the monocytes from asymptomatic patients showed IFN-γR down-regulation from 25.05 to 6.65 (Table1, experiment 2). The addition of neutralizing anti-IFN-γ antibodies slightly increased the IFN-γR expression to 9.54. In a seronegative patient (Table 1; experiment 5), the extent of down-regulation was smaller, with the MCF decreasing from 25.12 to 12.06, but the addition of anti-IFN-γ antibodies did not change the expression of IFN-γR significantly from 12.06 to 13.66. These results suggest that IFN-γ is partially involved in the down-regulation of the IFN-γR in HSV-2 seropositive patients, but it is not likely involved in HSV-2 seronegative patients, as these patients do not secrete IFN-γ after HSV-2 challenge because there is no immune memory response. After IFN-γ binding to its receptor there may be receptor internalization in patients previously infected with the virus (83). However, there may be other mechanisms at play in the regulation of receptor expression as suggested by the neutralization experiments.

Lack of effect of anti-IL10 neutralizing antibody on IFN-γR expression in HSV-2 seropositive and seronegative patients

IL-10 is believed to be an IFN-γ antagonist; therefore, the effect of IL-10 neutralization was examined on seropositive and seronegative patients. In this experiment, anti-IL-10 neutralizing antibody was incubated with UV-inactivated Ag and PBMC for 24hrs. After that the PBMC were collected and labeled with R-PE tagged IFN-γR Ab and FITC tagged CD14 Ab. IL-10 level of the supernatant in the overnight culture were checked by using ELISA.

In asymptomatic patients, neutralizing anti-IL-10 Ab did not inhibit the down-regulations of IFN-γR by HSV-2 Ag (Figure 5). The MCFs of the HSV-2 Ag exposed asymptomatic sample, before and after addition of anti-IL-10, were 4.93 and 4.94, respectively (Table 1; experiment 3). In HSV recurrent patients, HSV-2 Ag caused only a mild shift from 3.93 to 6.85 after the addition of neutralizing IL-10 Ab (Table 1; experiment 4). In seronegative patients, the addition of anti-IL-10 neutralizing Ab further decreased the expression of IFN-γR from a MCF of 12.06 to 9.27 (Table 1; experiment 5). These results suggest that IL-10 is not responsible or has little effect on the IFN-γR down-regulation induced by HSV-2 antigens.

Incubation of supernatants from HSV-2 stimulated PBMC with neuroblastoma and THP-1 cells has no effect on IFN-γ R expression

Although IFNγ had some effects on the down-regulation as it was previously shown in figure 1, it still cannot account for the entire effect of the down-regulation. The same result applied on IL-10. There might be a chance that other cytokines play a role in the regulation of IFNγR. To answer this question, supernatants of PBMC cultures that demonstrated inhibition of the IFN-γR expression were harvested and incubated with THP-1 or neuroblastoma cells.

These experiments demonstrated that the expression of IFN-γR on neuroblastoma and THP-1 cells was not affected by exposure to PBMC supernatants (Figures 5a, b). This suggests that soluble factors induced by HSV-2 in PBMC are unlikely to be involved in the regulation of IFN-γ R on THP-1 and neuroblastoma cells.

Incubation of supernatants from HSV-2 stimulated PBMC with Neuroblastoma and THP-1 cells

Figure 12a: THP-1 IFNγR expression

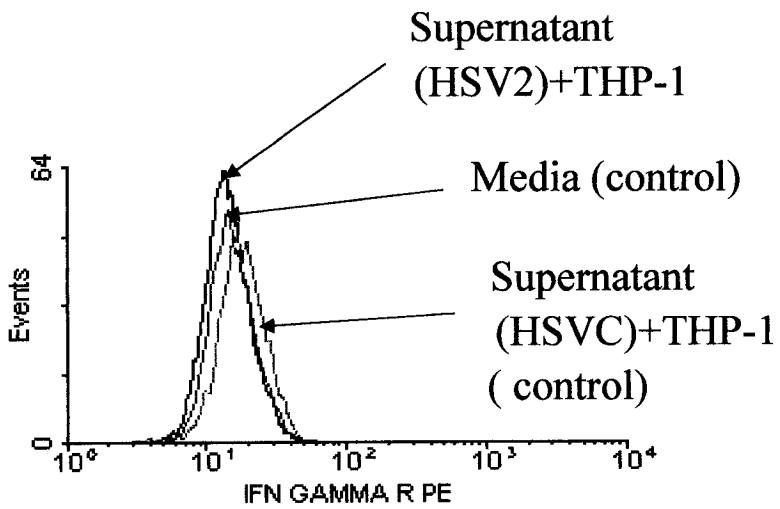


Figure 12 b: Neuroblastoma IFN-g R expression

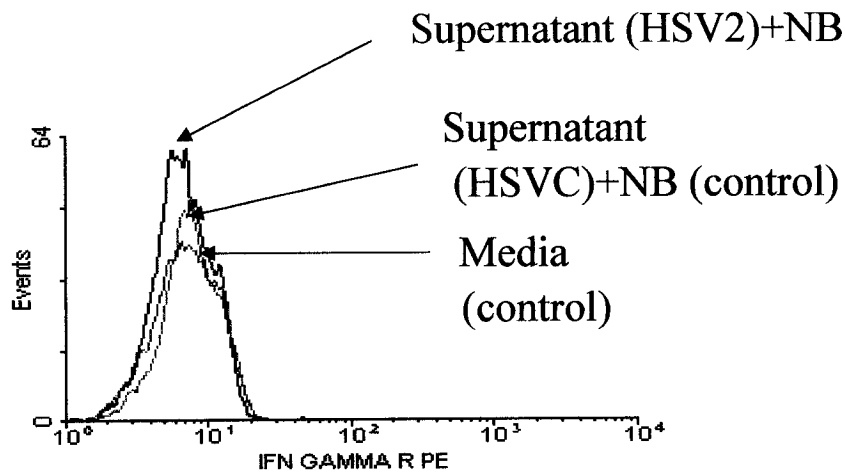


Figure 12 a, b:

Objective: To determine whether soluble factor contained in PBMC supernatants incubated with HSV-2 induce a change in the expression of the IFN-γR on THP-1 and Neuroblastoma cells.

Method: The supernatants harvested from PBMC cultures incubated with HSV-2 and controls were used as media for the THP-1 or neuroblastoma cell line cultures. After 24 hr. incubation, the cells were washed and stained with tagged monoclonal antibodies specific for IFN-γR.

Results: The expression of IFN-γR on THP-1 cells and neuroblastoma cells was not affected by exposure to PBMC conditioned supernatants. a) THP-1 and b) neuroblastoma cells.

Down-regulation of IFN-γR on monocytes is not caused by the secretion of soluble factors

In order to confirm and correctly interpret the results on the effect of PBMC supernatants on the IFN-γR expression in the cell lines, a more direct method was tested. Since the effective dosage and the viability of soluble factors after refrigeration were still questionable, another experiment was designed. For this purpose a semi-permeable membrane was used to separate HSV-2-stimulated PBMC from purified monocytes and THP-1 cells. The purified monocytes of seronegative patient were obtained by eliminating specific cell subpopulations by negative selection with magnetic beads. The objective of the experiment was to determine whether the monocyte IFN- γR down-regulation induced by exposure of PBMC to HSV-2 is caused by the secretion of soluble factors. The semi-permeable membrane allowed soluble factors produced by PBMC to travel and interact with the cell lines, disallowing cell to cell interaction. This system would detect a simultaneous down-regulation of IFN-γR in cells located in both chambers which are separated by the fluid permeable membrane. Comparing with the previous supernatant incubation experiment, this method is more direct and it is not affected by the concentration of soluble factors. The negative control included purified monocytes without PBMC in the co-culture. In the experimental arm, the purified monocytes or THP-1 cells were at the bottom of the plate with the semi-permeable membrane separating them from HSV-2 Ag stimulated-PBMC, which was located on the top well (Figure 13a).

The IFN-γR expression on purified monocytes was not influenced by soluble factors secreted from PBMC (Figure 13b). The IFN-γR MCF of the sorted monocyte co-

cultured with PBMC was 7.13. While the IFN-γR MCF of the negative control was 10.44 (net shift of MCF was 3.31). Similarly, PBMC from HSV-2 seronegative individuals incubated with HSV-2 had an IFN-γR MCF of 9.08 and the MCF of PBMC incubated with media was 17.62 (net shift of MCF was 8.54).

Compared to the control conditions, expression of IFN-γR on THP-1 cells did not change significantly after longer incubation times of 24 and 48 hours (figure 13c, d). These results suggest that soluble factors have little effect on IFN-γR expression in either purified monocytes or THP-1 cells.

Semi-permeable Membrane Experiment set up and IFN-γR expression on purified monocytes

Fig. 13a

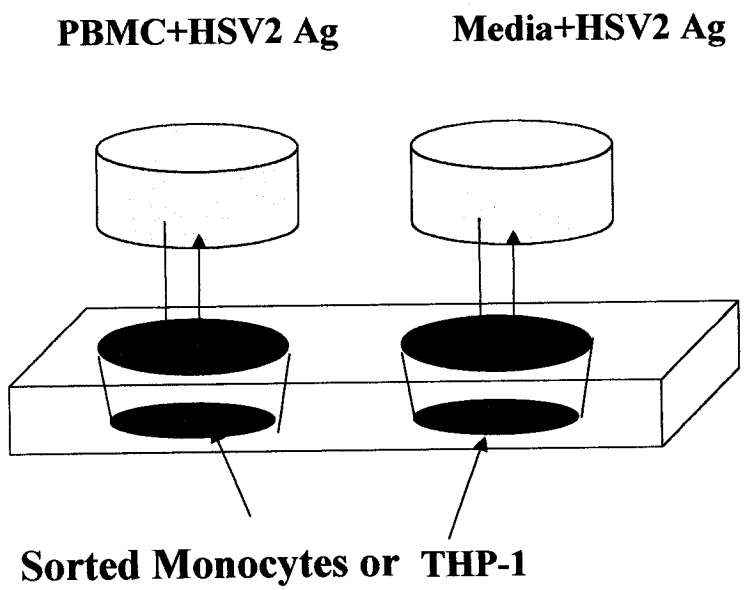


Figure 13b

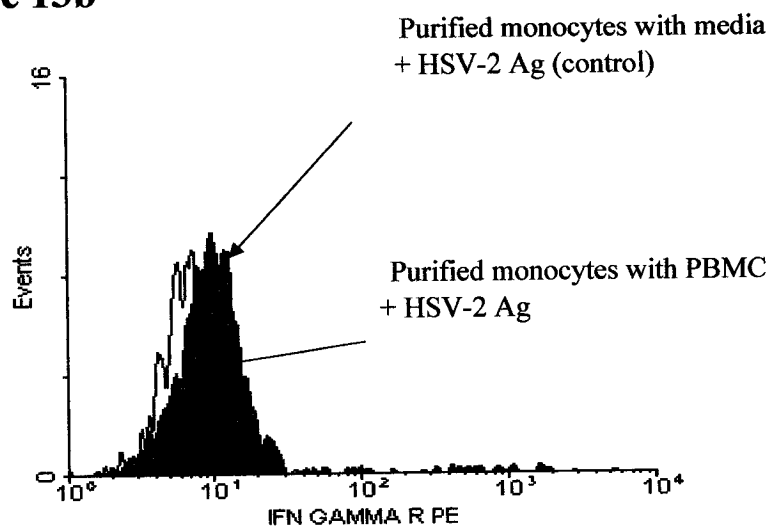


Figure 13.

Objective: To determine whether soluble factor play a role in the regulation of IFN-γR expression. In order to confirm and correctly interpret the results on the effect of PBMC conditioned supernatants on the IFN-γR expression in the cell lines, a more direct method was tested. Since the effective dosage and the viability of soluble factors after refrigeration were still questionable. For this purpose a semi-permeable membrane was used to separate HSV-2-stimulated PBMC from purified monocytes and THP-1 cells. The semi-permeable membrane allowed soluble factors but not cells to interact in each co-culture chamber. This system has the potential to detect a simultaneous down-regulation of IFN-γR in cells located in both chambers which are separated by a permeable membrane. The negative control was the purified monocytes without PBMC on the other side of culture plate. The purified monocytes or THP-1 were at the bottom of the plate with the semi-permeable membrane separating them from HSV-2 Ag stimulated-PBMC.

Figure 13a. Descriptive figure to illustrate the location of culture chambers, cells and semi-permeable membrane.

Figure 13b. This graph demonstrates the fluorescent intensity of the IFN-γR expressed on purified human monocytes co-cultured with PBMC incubated with HSV-2 or HSV-2 alone. There was no significant change between these two conditions, demonstrating that soluble factors are unlikely to mediate the HSV-2 induced effect on IFN-γR expression.

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Semi-permeable Membrane Experiment set up and IFN-γR expression in THP-1 at 24 and 48 hrs

Figure 13c.

THP-1

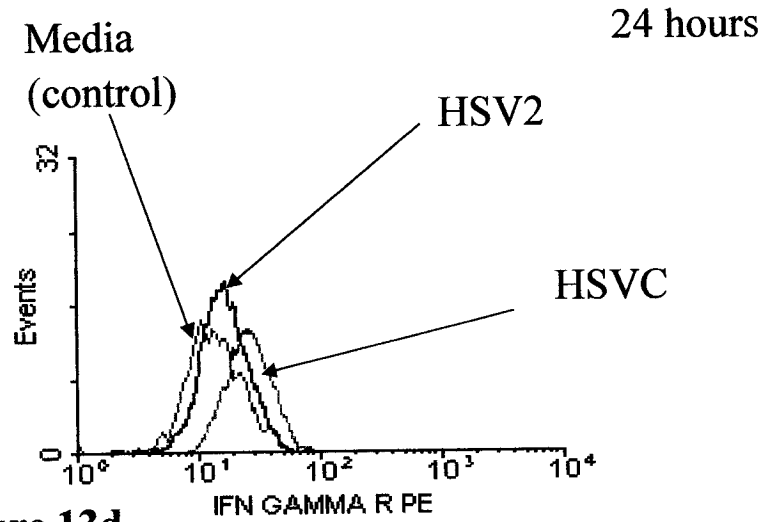


Figure 13d.

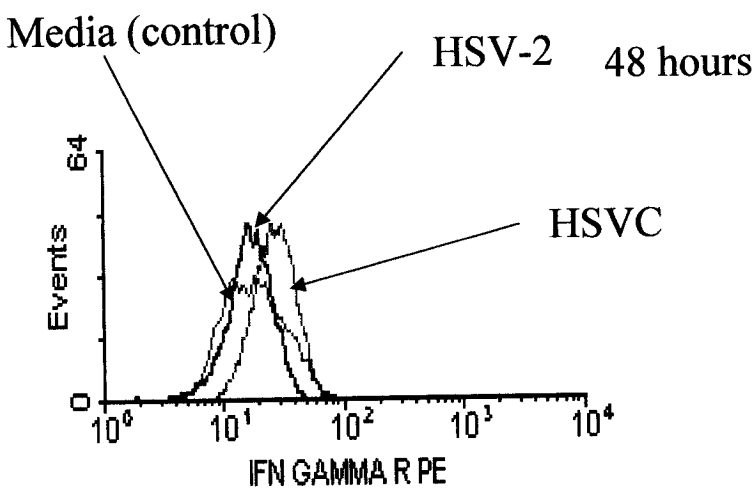


Figure 13c, d:

IFN-γR expression in THP-1 cells separated from HSV-2 stimulated PBMC by a semi-permeable membrane and incubated for c) 24 hours and d) 48 hours.

Cell-to-cell interactions as a mechanism of IFN-γR down-regulation

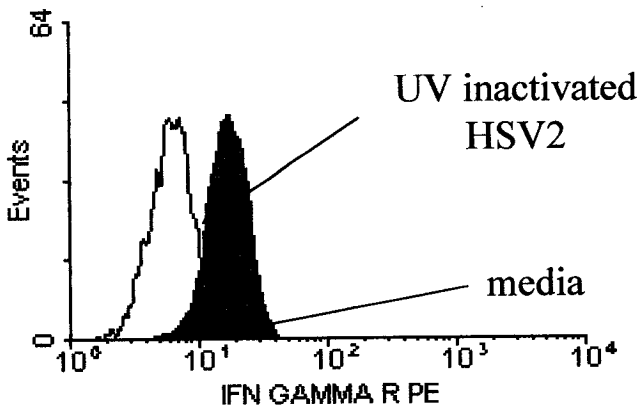
As soluble factors were not likely involved in the IFN-γR regulation, cell depletion experiments were performed to determine whether HSV-2 regulation of IFN-γR was mediated by cell-to-cell interactions. In the cell-depletion experiment, a number of patients were chosen who were HSV-2 seropositive or seronegative. PBMC were depleted of individual cell types one at a time by using magnetic beads. The depleted cell types included NK cells, B cells, and T cells. The cell depleted PBMC were then cultured with UV-inactivated HSV-2 Ag for 24 hrs. Then depleted PBMC were collected and labeled with Abs.

Table 2 summarizes MCF results of the depletion experiment, while typical experiments are shown in figure 14 (a, b, c). In PBMC from HSV-2 seropositive patients (n=6) the IFN-γR MCF of monocytes cultured in media and inactivated HSV-2 Ag was 16.64+/-4.79 and 8.83+/-2.16 (p=0.0019), respectively (Table 2). The MCF demonstrated down-regulation of IFN-γR in NK cell depleted PBMC incubated with HSV-2 when compared with the media control (Table 2 and Figure 14a). The IFN-γR MCF of NK depleted monocytes in media and UV inactivated HSV-2 Ag was 13.87+/-1.08 and 5.11+/-2.15 (p=0.065), respectively. The difference in IFN-γR expression before and after NK depletion was statistically significant (8.83+/-2.16 vs. 5.11+/-2.15, respectively, two-tailed t-test p= 0.05) (Table 2). The down-regulation in the absence of NK cells demonstrates that NK cells do not induce the down-regulation of the IFN-γR after HSV-2 exposure. However, the NK cells might have a role in antagonizing this down-regulation. As seen in the figure 8c, the depletion of NK cells caused further down-regulation after

HSV-2 exposure. These results should be interpreted with care, however, because there were only two individuals in the NK-depleted HSV-2-seropositive group and it is possible that the results may be skewed. However, these results are likely significant, as a statistical difference was demonstrated in such a small sample. A similar experiment was done with PBMC depleted of B cells. Despite B cell depletion, HSV-2 induced IFN-γR down-regulation (Table 2 and Figure 8b). IFN-γR expression in PBMC depleted of B cells and incubated in media alone from seropositive patients decreased from 18.98+/-0.16 to 7.42+/-1.37 ($p=0.0003$) after incubation with inactivated HSV-2 (Table 2).

How IFN-γR downregulation is affected by NK cell depletion

Figure14a PBMC



PBMC minus NK cells

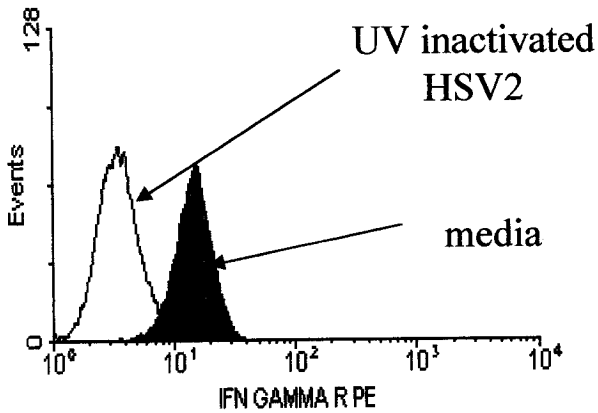


Figure 14a:

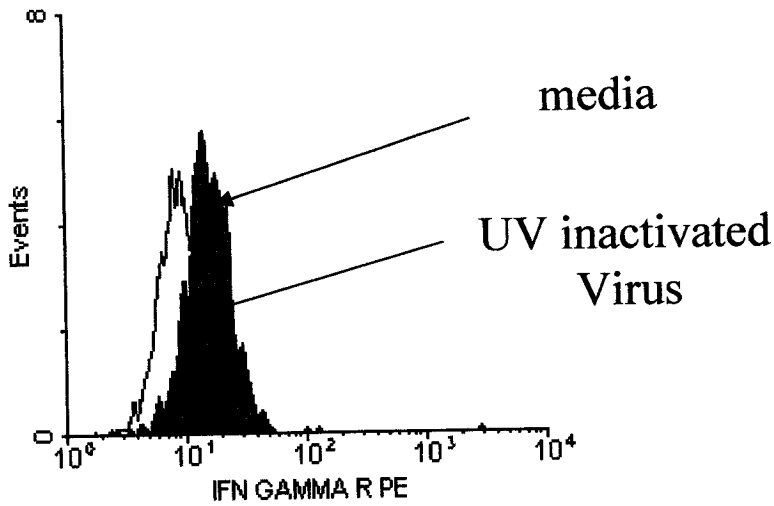
Objective: Cell depletion experiments were performed to determine whether the down-regulation of IFN-γR observed in monocytes after HSV-2 antigen exposure was mediated by cell-to-cell interactions.

PBMC were depleted of NK cells, which where then incubated with HSV-2 or media and the IFN-γR expression was measured by flow cytometry.

How IFN-γR down-regulation is affected by B cell depletion

Figure14b

PBMC



PBMC-B cell

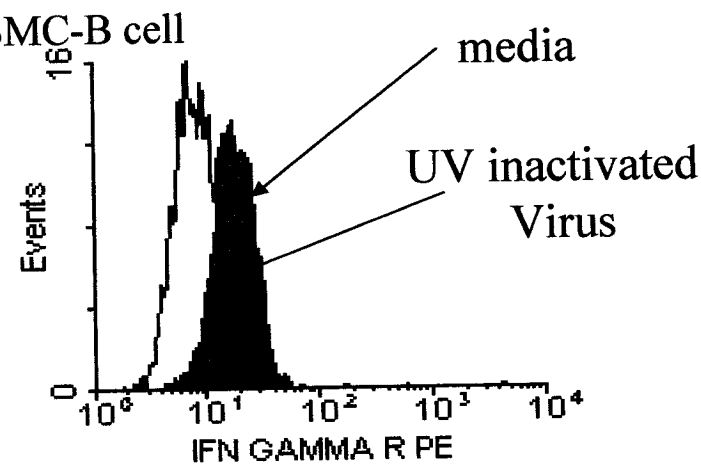


Figure 14b:

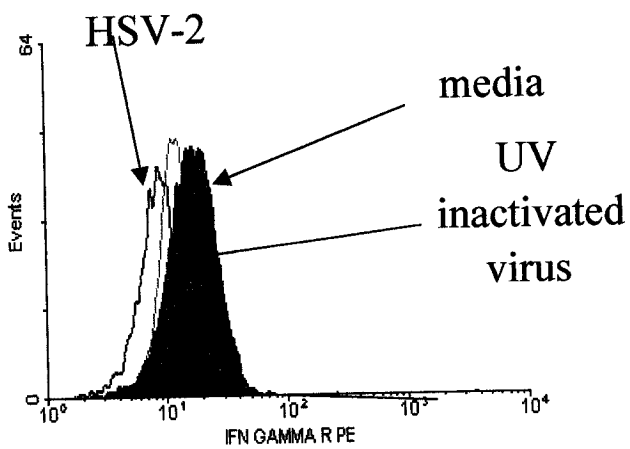
Objective: Cell depletion experiments were performed to determine whether the down-regulation of IFN-γR observed in monocytes after HSV-2 antigen exposure was mediated by cell-to-cell interactions.

PBMC were depleted of B cells, which where then incubated with HSV-2 or media and the IFN-γR expression was measured by flow cytometry.

How IFN-γR downregulation is affected by T cell depletion

Figure 14c

PBMC (undepleted)



PBMC-T cell

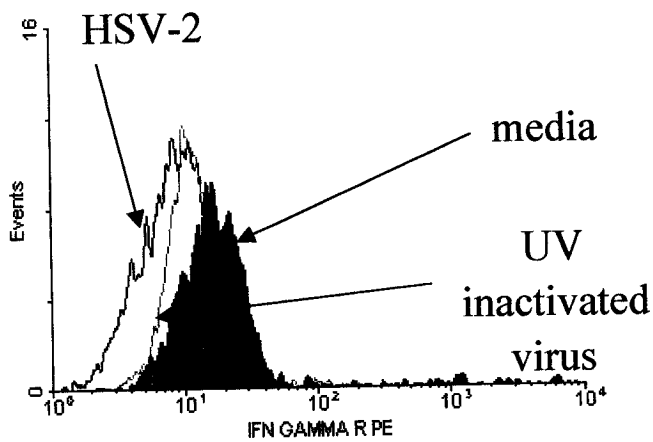


Figure 14c:

Objective: Cell depletion experiments were performed to determine whether the down-regulation of IFN-γR observed in monocytes after HSV-2 antigen exposure was mediated by cell-to-cell interactions.

PBMC were depleted of T cells, which where then incubated with HSV-2 or media and the IFN-γR expression was measured by flow cytometry.

Table 2: Flow cytometry result of IFN-γR on monocytes PBMC depleted of individual cell types, and the expression of IFN-γR was then determined after culture with HSV-2.

The mean channel fluorescence in the corresponding cell depleted population					
On $CD14^+$cells	**PBMC**				
	M	HSV-2	P(MvsHSV)	P(HSV; pos vs neg)	
	MCF+/-SD	MCF+/-SD			
Seropositive	16.64+/-4.79	8.83+/-2.16	p=0.0019		N=6
Seronegative	16.27+/-3.25	9.47+/-0.97	p=0.000421	0.3	N=5
	NK cell depleted PBMC				
	M	HSV-2	P(MvsHSV)	P(PBMCvsNK)	
	MCF+/-SD	MCF+/-SD			
Seropositive	13.87+/-1.08	5.11+/-2.15	p=0.065	p=0.025	N=2
Seronegative	NIL	NIL			
	B cell depleted PBMC				
	M	HSV-2	P(MvsHSV)	P(PBMCvsB)	
	MCF+/-SD	MCF+/-SD			
Seropositive	18.98+/-0.16	7.42+/-1.37	P=0.000343	p=0.12	N=2
Seronegative	39.12	6.23			N=1
	T cell depleted PBMC				
	M	HSV-2	P(MvsHSV)	P(PBMCvsT)	
	MCF+/-SD	MCF+/-SD			
Seropositive	16.97+/-2.87	11.56+/-3.49	p=0.056	p=0.035	N=3
Seronegative	20.01	17.38			N=1

Objective: To determine whether the HSV-2 induced down-regulation of IFN-γR observed in monocytes was mediated by cell-to-cell interactions. PBMC samples were collected from several patients. PBMC were depleted of individual cell types, and the expression of IFN-γR was then determined on monocytes after culture with HSV-2. IFN-γR expression on monocytes is measured as mean channel fluorescence (MCF) by flow cytometry using a phycoerythrin labeled monoclonal antibody and a FITC labeled monoclonal antibody (α-CD14) that recognizes monocytes only. The experiments were conducted by incubating PBMC or T- cell, B-cell, and NK-cell depleted HSV-2 seropositive and seronegative patient PBMC with media or HSV-2 inactivated virus. **M** = medium alone, **HSV2** = culture purified HSV-2, **Inact virus** = heat or UV inactivated HSV-2. (M vs. HSV) p value between medium and heat inactivated culture; (PBMC vs NK) p value between the cells exposed to HSV-2 before and after depletion of NK cells; (PBMC vs. B) p value between the cells exposed to HSV-2 before and after depletion of T cells; (HSV; pos. vs. neg) p value between the set of data of HSV-2 seropositive and seronegative patients. N = sample size

Statistical analysis

The MCF in table 2 is the average of different samples and SD represents the standard deviation. The p value is calculated by using two-tailed t-test between groups. P value is significant when he value is ≤ 0.05.

Similar IFN-γR expression occurred after whole PBMC and PBMC depleted of B cells were incubated with HSV-2 (there was no difference in the MCF values between these groups, p=0.12). PBMC expression of IFN-γR decreased from 16.64+/-4.79 to 8.83+/-2.16 after incubation with purified UV inactivated virus, resulting in a net shift in MCF of 7.81 (Table2). The corresponding changes in B cell depleted samples of PBMC shifted from 18.98+/-0.16 to 7.42+/-1.37, which is a net decrease in MCF of 11.57 (Table 2). Hence, down-regulation of IFN-γR occurred in both undepleted and B cell depleted PBMC. There was a trend towards a greater down-regulation of monocyte IFN-γR in the B cell depleted PBMC than in T cell depleted PBMC. In PBMC depleted of T-cells, MCF for the monocytes incubated with HSV-2 was 11.56+/-3.49 vs. 7.42+/-1.37 in the media control, two-tailed t test p=0.056. The depletion of B cells may have facilitated the down-regulation of the IFN-γR. Therefore, depletion of T cells had an important impact on the regulation of IFN-γR by HSV-2. At least part of the T cell effect on IFN-γR expression in cells from seropositive volunteers may be due to their ability to secrete interferon gamma after memory cell stimulation with HSV-2. The down-regulation of the receptor may be due to its recognition by ligand followed by its internalization in HSV-2 seropositive patients. However, other unknown elements may be at play, such as cell-to-cell interaction through their T cell receptor and MHC II class binding as well as other surface ligand-receptor interactions (i.e.: B7/CD28, ICOS, etc.). In addition, the comparison of the receptor MCF in whole PBMC and the PBMC depleted of T cells cultured with HSV-2 (8.83+/-2.16 vs. 11.56+/-3.49, p=0.035), demonstrated a net increase in IFN-γR expression, suggesting a derepression of the receptor in the absence of T cells.

In PBMC from HSV seronegative patients, IFN-γR expression was reduced from 16.27+/-3.25 to 9.47+/-0.97 (p=0.0004) after HSV-2 incubation, a net decrease of 6.80 in non-depleted PBMC (Table 2). There was no difference in monocytic IFN-γR expression after HSV-2 incubation between HSV-2 seropositive and HSV-2 seronegative subjects (8.83+/-2.16 vs. 9.47+/-0.96, two-tailed t-test, p=0.3). After B cell depletion, HSV-2 antigen induced an IFN-γR expression drop from 39.12 to 6.23 (Table 2).

In HSV-2 seronegative T cell depleted PBMC, HSV-2 Ag induced an IFN-γR expression drop from 20.01 to 17.38 (net shift of 2.6). This change was minor and unlikely to be significant (Table 2).

It was particularly interesting to find a facilitatory role for NK cell depletion in the down-regulation of IFN-γR. NK cell might stabilize and resist the changes of the IFN-γR expression on the monocyte surface. Therefore, the IFN-γR might become more susceptible to down-regulation in patients who are defective in NK cell. This observation is novel and may explain the increased susceptibility to HSV-2 recurrent disease in patients who have low NK cell function (90). Decreased NK cell number or function in this susceptible sub-population of patients may cause lower levels of IFN-γR expression and a decreased antiviral effect of interferon gamma. In contrast, T cell depletion had an inhibitory role in the down regulation of the IFN-γR. Similarly, the comparison of T cell depleted and whole PBMC experiments demonstrated that HSV-2 induced an increase in IFN-γR expression after T cell depletion, suggesting again that T cells contribute to the down-regulation of IFN-γR on monocytes.

Furthermore, the effect of HSV-2 on IFN-γR expression on purified monocytes was investigated (Table3). In this experiment, five patients were chosen from both

Table 3: Flow cytometry result of IFN-γR expression on human purified monocytes or PBMC with exposure to inactivated HSV-2.

The mean channel fluorescence in both seropositive and seronegative patient						
Exposed with non-purified HSV-2 Ag and purified HSV-2 Ag						
	HSV2-Seropositive					
	Media	HSV-2	p(MvsHSV)	p(HSV2s)	Sample size	
	MCF+/-SD	MCF+/-SD				
PBMC	16.67+/-5.17	8.57+/-2.17	p=0.005078	p=0.17	N=5	
Monocyte	15.34+/-3.21	10.99+/-3.85	p=0.04		N=5	
	HSV-2 SeroNegative					
	Media	HSV-2	p(MvsHSV)			
	MCF+/-SD	MCF+/-SD				
PBMC	16.27+/-3.25	9.47+/-0.97	p=0.000421	p=0.12	N=5	
Monocyte	10.94+/-5.45	11.95+/-3.96	p=0.67		N=5	
MCF:	Mean channel fluorescence					
SD:	Standard deviation					

Objective:

IFN-γR expression on human purified monocytes or PBMC was investigated after incubation to inactivated HSV-2.

IFN-γR expression was measured as mean channel fluorescence (MCF) by flow cytometry of purified monocytes. Monocytes were purified by negative selection and MCF was compared between whole PBMC and sorted monocytes after exposure to inactivated HSV2.

IFN-γR expression on monocytes is measured as MCF by flow cytometry using a phycoerythrin labeled monoclonal antibody (IFN-γR) and a FITC labeled monoclonal antibody (α-CD14) that recognizes monocytes only. Positive= seropositive patient (either recurrent or asymptomatic), negative= seronegative patient, MCF= mean channel fluorescence, SD=standard deviation, (M vs HSV)=p-value between the data media vs. HSV; (HSV2s) p-value between MCF of the PBMC and purified monocyte sample data upon HSV-2 exposure, PBMC= peripheral blood nuclear cell, monocyte= purified monocytes.

The MCF in table 3 is the average value of several samples collected from different patients and SD represents the standard deviation. The p value is calculated by using two-tailed t-test between groups. P value was significant if ≤ 0.05.

seropositive and seronegative groups. Their monocytes were purified from PBMC by using negative selection with magnetic beads. Then the purified monocytes were cultured with UV-inactivated HSV-2 Ag for 24 hrs. Controls included purified monocytes cultured with media only. Purified monocytes were collected and labeled with Abs. Monocytes from whole PBMC from HSV-2 seropositive and seronegative demonstrated down-regulation of IFN-γR after culturing with HSV-2, (media vs HSV-2, p=0.005 and 0.004, respectively). Similarly, sorted monocytes from HSV-2 seropositive patients expressed less IFN-γR in response to UV- inactivated HSV-2 Ag (p=0.04, table 3). However, there was a no IFN-γR down regulation in sorted monocytes of HSV-2 seronegative patients (p=0.67, table 3). These results demonstrate that the IFN-γR is down regulated in both isolated and non-isolated monocytes upon exposure to inactivated HSV-2 in HSV-2-seropositive patients. Cell-to-cell interactions had little or no influence in the down-regulation of IFN-γR in HSV-2 seropositive patients. In contrast, the HSV-seronegative patient had down-regulation of the IFN-γR in the PBMC but not in the isolated monocytes. This suggests that monocytes from HSV-2 seronegative patients require an innate immune response and IFN-γR regulation is dependent on cell-to-cell interaction. These results suggest that an acquired immune response or an innate mechanism has influence on how the IFN-γR is regulated by HSV-2. It seems that the regulation of IFN-γR in HSV-2 seronegative patients is IFN-γ independent. This is supported from these results and also from those obtained with the IFN neutralizing antibody represented in Figure 1b. It is suggested that the regulation of IFN-γR is different in HSV-seronegative individuals. It is also likely that individual differences exist among individual in the regulation of this molecule.

Inhibition of CD28/B7 interaction has no effect on IFN-γR down-regulation

From the previous depletion experiment, it was found that T cells facilitate in certain extent in down-regulation of IFN-γR in seropositive patient (Table 2). In seronegative patients, T cell contributes to a larger extent in down-regulation of IFN-γR. Therefore, it would be interesting to know which receptor interact between T cells and monocytes, CD28/B7 would be the target. In order to investigate the effect or associations between CD28 and B7 on monocytic IFN-γR, CTLA-4 was used to block interactions between these co-stimulatory molecules (4). CD28/B7 interaction sends an important co-stimulatory signal to T cells required for activation, resulting in the secretion of IL-2. CTLA-4 concentrations of 1, 2, and 4 μg/ml were used to examine the inhibition of the CD28/B7 interaction. CTLA-4 was incubated with UV-inactivated HSV-2 Ag and PBMC for 24 hrs. Then PBMC were collected and labeled with Ab, and CTLA-4 effect on IFN-γR expression was examined. Results suggested that blocking of CD28/B7 interactions has no significant effect on the IFN-γR down-regulation induced by HSV-2 (Figure 15a). As it can be seen, the IFN-γR down-regulation in HSV-2 Ag exposed samples was not affected by the increased concentration of the CTLA-4 (Figure 15a). On the other hand, CD80 and CD86 expression decreased after exposure to HSV-2 and, as expected, it demonstrated an inverse relationship with CTLA-4 in a dose response experiment (Figure 15b, c). As another control, IL-2 secretion in supernatants was also monitored and in the absence of CTLA-4, IL-2 concentration was 804 pg/ml. With the addition of 1 μg CTLA-4, the IL-2 concentration was reduced to half of its original value (435 pg/ml). In the presence of 2μg/ml and 4μg/ml CTLA-4 fusion protein, IL-2 concentration was similar to that observed for 1 μg/ml of CTLA-4 (Figure 15d).

Therefore to summarize results of the cell-cell interaction experiments, it appears that the regulation of monocytic IFN-γR in cells in seropositive patients is significantly influenced by NK cells or T cells since depletion of these cells did have a significant, but opposing effects on its expression. For instance, the largest down-regulation on the IFN-γR in the cell depletion experiments was observed after the depletion of NK cells. As it was discussed above, depletion of these cells had a facilitatory role in the down-regulation of IFN-γR induced by HSV-2. However, the mechanism by which NK cells work in this situation is unknown. In contrast, deletion of T-cells had an inhibitory role in the HSV-2 induced effect on IFN-γR. As discussed, this effect may be explained by the secretion of IFN-γ. However, the experiments with the neutralizing antibodies negate a large contributory effect on this effect. It was for this reason that the involvement of CD28 and B7 was investigated. Although, this monocyte/T-cell interaction results in the delivery of a signal to the CD28 molecule, it is also probable that there is a signal delivered to the monocyte during this interaction (82). However, blocking CD28 had little influence on the regulation of IFN-γR induced by HSV-2. It is highly likely that T cells play a key role in the regulation of the IFN-γR through both IFN-γ dependent and independent mechanisms. In this last instance, a cell-cell interaction is required. However, this mechanism is unknown. B-cells may have a lesser function or no role in IFN-γR regulation.

Effect of CTLA-4 on IFN-γR and CD80 expression after stimulation of HSV-2 seropositive PBMC with HSV-2 Ag

Figure 15a

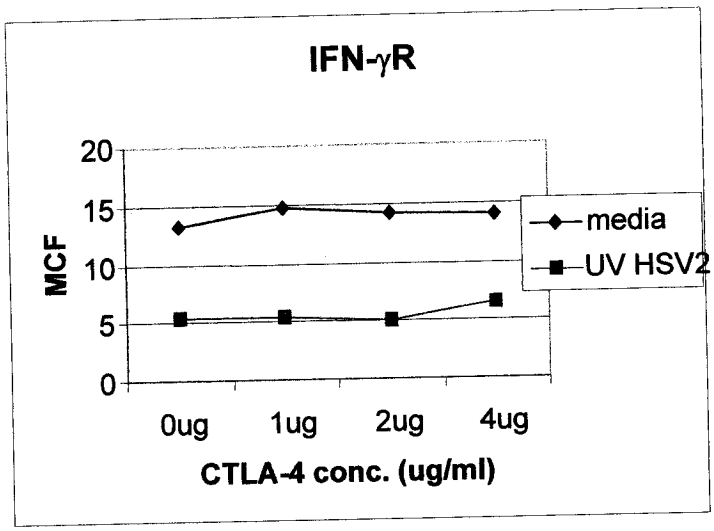


Figure 15b

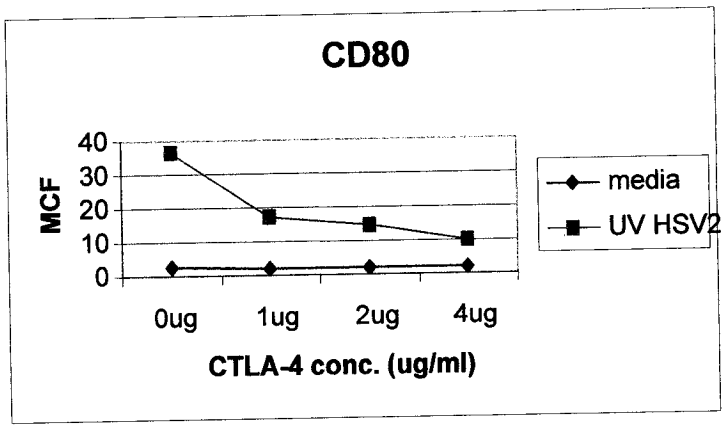


Figure 15a, b:
Objective:

To investigate the effect of CD28 and B7 interaction on HSV-2 induced regulation of monocytic IFN-γR and on the expression of CD80 (B7-1), and CD86 (B7-2). CD28/B7 interactions were blocked by CTLA-4.

a). IFN-γR, b) CD80 expression were measured by flow cytometry as mean channel fluorescence (MCF) following HSV-2 or media incubation and the addition of CTLA-4. CTLA-4 1, 2, and 4 μg/ml were used to block interactions between these co-stimulatory molecules.

Result:

CTLA-4 has no significant effect on the IFN-γR down-regulation induced by HSV-2. On the other hand, the increase of CTLA-4 dosage can cause the decrease of CD80 and CD86 expression in the presence of HSV-2.

Effect of CTLA-4 on CD86 expression and IL-2 secretion after stimulation of PBMC with HSV-2 Ag (Seropositive patient)

Figure 15c

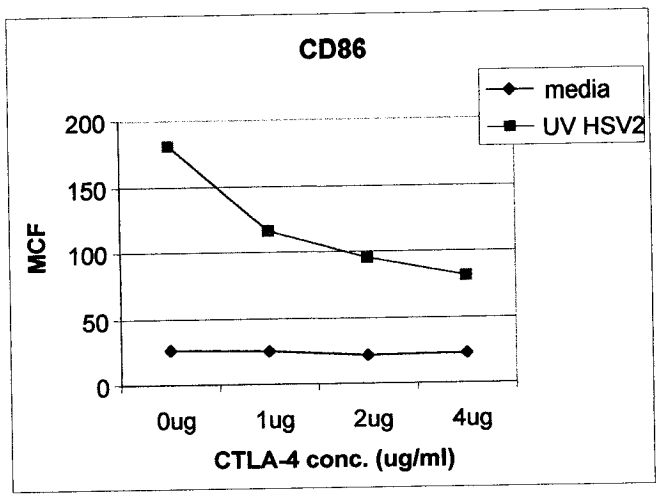


Figure 15d

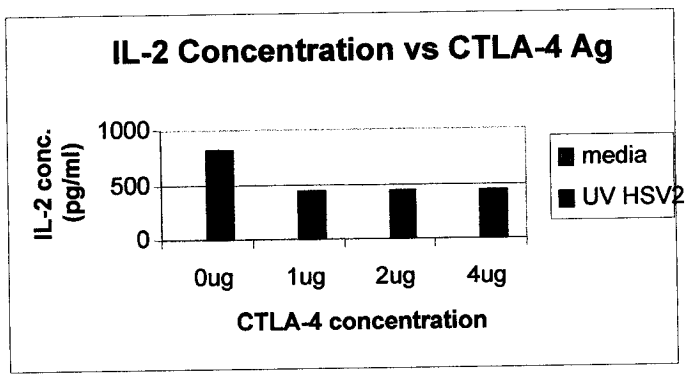


Fig 15 c, d

Investigate the effect of CD28 and B7 interaction on HSV-2 induced regulation of monocytic CD86 (B7-2) expression. CD28/B7 interactions were blocked by CTLA-4. After the blocking of CD28/B7 interaction, IL-2 secretion were measured by ELIZA

Figure 15c) CD86 expression was measured by flow cytometry as mean channel fluorescence (MCF) following UV-inactivated HSV-2 or media incubation and the addition of CTLA-4. CTLA-4 1, 2, and 4 μg/ml were used to block interactions between these co-stimulatory molecules. d) Supernatant IL-2 concentration with the addition of CTLA-4

Table 4: The effects of CTLA-4 on the regulation of monocytic IFN-γR CD80, CD86 and IL-2 expression.

Table 4		IFN-γR				CD86	
		MCF	MCF			MCF	MCF
	CTLA-4	media	UV HSV-2		CTLA-4	media	UV HSV-2
	0ug	13.18	5.44		0ug	26.96	181.62
	1ug	14.82	5.46		1ug	25.48	116.53
	2ug	14.16	4.96		2ug	22.1	94.38
	4ug	14.04	6.6		4ug	22.88	80.84
		CD80					
		MCF	MCF			Conc IL-2 pg/ml	
	CTLA-4	media	UV HSV-2		CTLA-4	media	UV HSV-2
	0ug	2.89	36.6		0ug	0	804
	1ug	2.1	17.32		1ug	0	435
	2ug	2.1	14.93		2ug	0	440
	4ug	2.22	10.29		4ug	0	440

PBMC were incubated with UV inactivated HSV-2 or media control. The expression of IFN-γR, CD80, CD86, and IL-2 was analyzed following the addition of increasing doses of CTLA-4.

The down-regulation of IFN-γR mediated by HSV-2 does not require de novo protein synthesis

To analyze the effect of *de novo* protein synthesis on IFN-γR expression, two different doses of cycloheximide (CHX) were tested. CHX was cultured with UV-inactivated HSV-2Ag or the non-purified heat inactivated HSV-2 Ag and PBMC for 24hrs. PBMC were collected and labeled with R-PE tagged IFN-γR and FITC tagged CD14 Ab. The effect of CHX on IFN-γR expression was investigated by flow cytometry. Previous work on CHX showed that 2μg/ml is sufficient to inhibit protein synthesis in PBMC (91). Also, these studies indicated that incubation with CHX should be less than 24 hrs to avoid cell death (92). Results showed that the extent of IFN-γR down-regulation with inactivated HSV-2 was the same for cells incubated with 1 μg/ml and 2 μg/ml CHX (Figure 16a and Figure 16b). These results suggest that the IFN-γR down-regulation by HSV-2 does not require *de novo* protein synthesis.

IFN-γR expression is not regulated at the mRNA level

To determine whether IFN-γR regulated at mRNA level, microarray was done to check the change of IFN-γR gene expression. PBMC was cultured with UV-inactivated HSV-2 Ag for 24 hrs and then monocytes were purified from PBMC. RNA of the purified monocyte was extracted. Filter cDNA microarrays were used to examine changes in gene expression in purified monocyte after HSV-2 incubation. In HSV-2 seropositive patients, IFN-γR1 mRNA expression did not change in monocytes following 24 hour incubation of PBMCs with HSV-2. However, there was a slight increase in the level of IFN-γR1 mRNA expression in HSV-2 seronegative patients, from 0 to 90.76 (the

number is a relative measure of signal intensity) (Table 5) and the corresponding ratio of mRNA expression level between UV inactivated HSV-2 Ag exposed sample to media control is 1.6 to 1. IFN-γR2 mRNA expression was increased in both HSV-2 seropositive and seronegative patients, with increases of 574.025 and 256.76, respectively. The corresponding ratio of IFN-γR2 mRNA expression level between UV- inactivated HSV-2 Ag exposed samples to media control is 1:1 for seropositive and 1.6:1 for seronegative patient sample. According to some microarray studies, if the experimental result changes are less than 3 fold, the change will be considered as insignificant (93). Therefore, the observed increases in optic density measures for the mRNAs may be within the variability range of the assay and may not be significant. These results imply that the HSV-2 Ag mediated IFN-γR down-regulation is not mediated by changes in its mRNA expression.

The effect of cycloheximide in the IFN-γR down-regulation induced by both UV-inactivated and non purified HSV-2 Ag

Figure 16a:
UV inactivated HSV-2

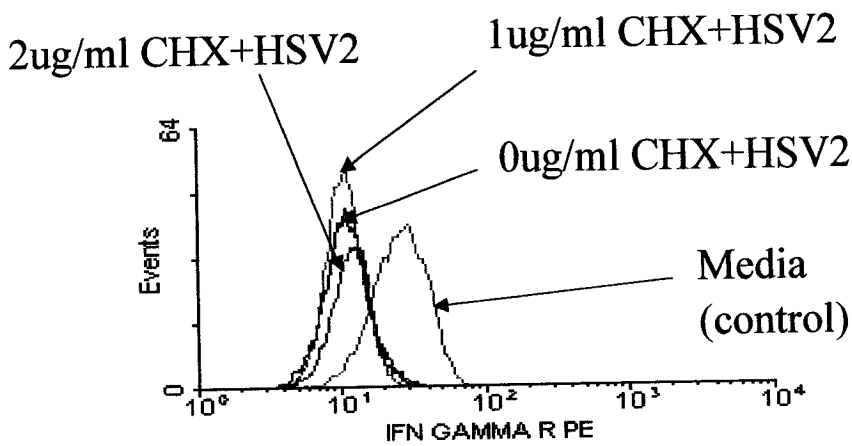


Figure 16b: Non-purified HSV2

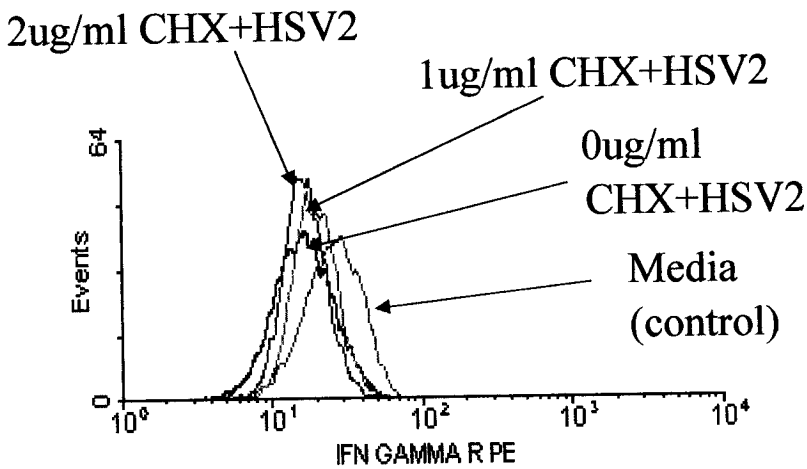


Figure 16a, b.

Objective:
To analyze the effect of *de novo* protein synthesis on IFN-γR expression, two different doses of cycloheximide (CHX) were tested (1 μg/ml and 2 μg/ml CHX).

IFN-γR expression in PBMC incubated with a) UV inactivated HSV-2 and b) non-purified HSV-2 following pre-incubation with cyclohexamide.

Results showed that the extent of IFN-γR down-regulation with inactivated HSV-2 was the same for cells incubated with 1 μg/ml or 2 μg/ml CHX

Table 5 Filter microarray results: Interferon gamma cytokine, interferon receptor gamma receptor chain 1 and interferon receptor gamma receptor chain 2 gene expression in both seropositive and seronegative patient (sample size n=3 for each group)

	Seropositive		Seronegative	
Gene Name	HSV2-Control Net changes	HSV2: Control ratio	HSV2-Control Net changes	HSV2: Control ratio
IFN-γ	0	1:1	107.57	1.6:1
IFN-γ R1	0	1:1	90.76	1.6:1
IFN-γ R2	574.025	1:1	256.765	1.6:1

Objective:

To examine whether the HSV-2 induced regulation of IFN-γR is due to transcriptional regulation of its mRNA. Filter cDNA microarrays were used to examine changes in gene expression after HSV-2 incubation.

Monocytes were purified after PBMCs were incubated with or without HSV-2. Monocyte RNA was reverse transcribed and labeled followed by hybridization to the microarray filter.

HSV2-control net change represents the difference of mRNA level between monocytes with PBMC cultured with UV-inactivated HSV-2 and media control. Ratio of mRNA of HSV2: control represents the ratio between the mRNA level of monocytes with PBMC culture with UV inactivated HSV-2 and control media.

There was a slight increase in the level of IFN-γR1 mRNA expression in HSV-2 seronegative patients, from 0 to 90.76 (the number is a measure of intensity).

IFN-γR2 mRNA expression was increased in both HSV-2 seropositive and seronegative patients, with increases of 574.025 and 256.76, respectively. The observed increases in optic density measures for the mRNAs may be within the variability range of the assay and may not be significant. These results may imply that the HSV-2 Ag mediated IFN-γR down-regulation is not mediated by changes in its mRNA expression.

Aim 3. - To assess the physiological impact of the IFNγR expression inhibition by HSV-2 on the IFNγ signaling pathway.

HSV-2 induces defective IFN-γ mediated signaling in HSV-2-seropositive individuals

Experiments were done to investigate whether HSV-2 induced IFN-γR down-regulation has an impact on the signaling pathway used by IFN-γ. It is known that IFN-γR1 associates with Janus kinase JAK1, and IFN-γR2 associates with JAK2. In addition, IFN-γ induces oligomerization of the IFN-γ receptor subunits, which leads to the transphosphorylation and activation of JAK1 and JAK2. The activated JAKs then phosphorylate Y440 of IFN-γR1, creating a docking site for signal transducer and activator of transcription STAT1. While bound to the receptor, STAT1 is phosphorylated on Y701 and is released from the receptor, forming a homodimer that translocates to the nucleus (59). Western blots were used to examine STAT-1 phosphorylation in the monocyte IFN-γ pathway. With the Western Blot method used, phosphorylated STAT-1 exists in 2 forms, which are demonstrated by bands of 91 and 84 kDa.

The PBMC collected from two seropositive patients were incubated with either media or UV- inactivated HSV-2 Ag in concentration of 1000:1 (viral particles to PBMC) for 24 hrs. After the incubation, the monocytes were purified from the cultures by negative selection with magnetic beads. There would be two sets of monocytes, one collected from the HSV-2 incubated PBMC and the other is the media incubated PBMC. The two sets of monocytes will be further split into two aliquots each. One part of each set was added with 200ng/ml recombinant IFN-γ to stimulate the IFN-γ pathway, the

other incubated in media. After 20min, these cells in the 4 cultures were lysed and analyzed by western blot for phosphorylation of STAT-1. The phosphorylation level of STAT-1 and its corresponding protein level were determined. This experiment was also performed in HSV-2 seronegative patients.

Results in Figure 17 demonstrate that STAT-1 from monocyte for HSV-2 seropositive and seronegative patients is poorly or it is not phosphorylated in the absence of IFN-γ (column 2, 4; Figure 17 a, c). and that monocytes from an HSV-2 seropositive patient have a STAT-1 phosphorylation deficit after HSV-2 incubation. In HSV-2 seronegative monocytes, the presence of a band of higher intensity than that of the media control sample indicates that recombinant IFN-γ induced greater STAT-1 phosphorylation even in the presence of HSV-2 than in its absence (Figure 17a). Densitometry measurement demonstrated that the intensity of the bands were 22.5% and 77.5% (% of density units) in media and UV-inactivated HSV-2 Ag both with IFN-γ exposed samples, respectively. The blot was stripped and re-probed with anti-STAT-1 antibody to ensure that the STAT-1 protein level was the same in all samples (Figure 17b) (Table 6). Densitometric analysis of STAT-1 band intensity showed that these bands were 15.94% and 17.28% in samples in media cultured with and without IFN-γ, respectively. The band intensities of UV-inactivated HSV-2 Ag exposed monocytes, with and without IFN-γ, were 36.84% and 29.94%, respectively. The intensity ratios of STAT-1 phosphorylation were 1.41 in unstimulated samples and 2.1 in samples exposed to HSV-2 (Table 6). These results suggest that STAT-1 protein phosphorylation increases upon exposure to HSV-2 in monocytes from HSV-2 seronegative patient, suggesting that HSV-2 is unable to alter the IFN-γ pathway under these conditions.

Figure 17 a, b

STAT1 protein +/- HSV-2 stimulation in Seronegative patient +/- IFN-γ

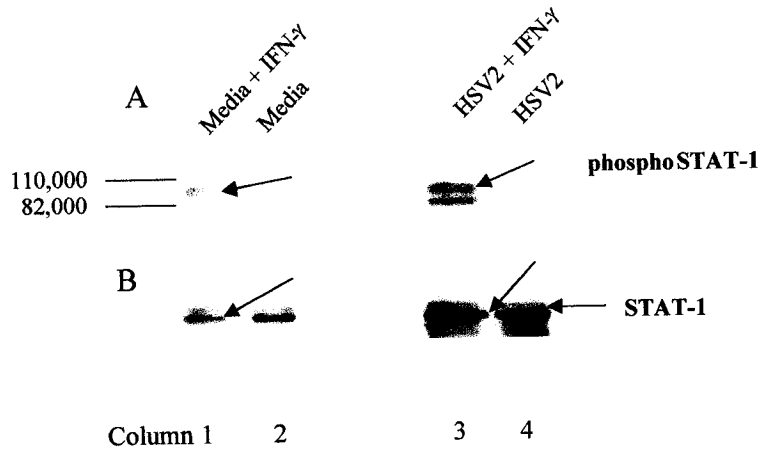


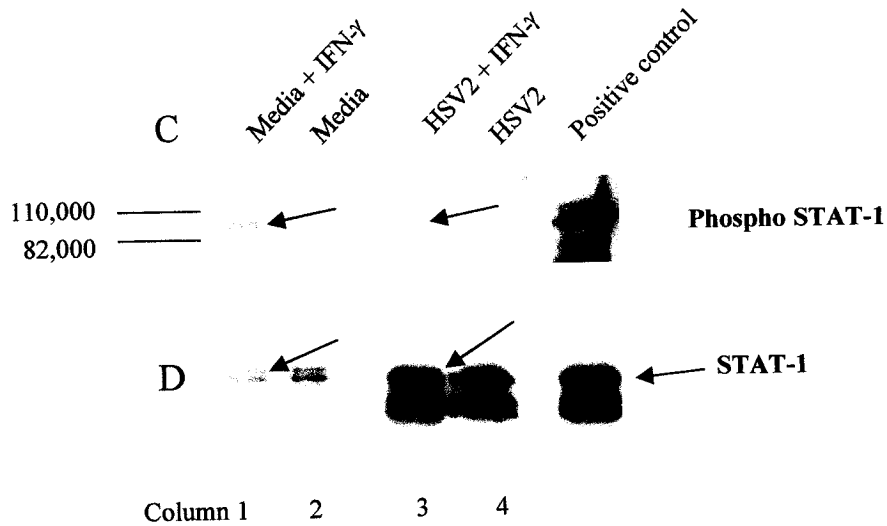
Figure 17A, B, C, D

Objective:
To investigate whether HSV-2 induced IFN-γR down-regulation has an impact on the signaling pathway used by IFN-γ.

Western blots and antibodies specific for STAT molecules were used to examine total concentration of STAT-1 and its relative ratio of phosphorylation in monocytes before and after IFN-γ and/or/HSV-2 stimulation. A) HSV-2 seronegative and C) HSV-2 seropositive patients. Arrows point to bands showing STAT-1 phosphorylation at Tyr 701. B, D) Band intensity indicates the level of STAT-1 identified using anti-STAT-1 antibody. This experiment was repeated three times.
STAT-1 protein phosphorylation increases upon exposure to HSV-2 in monocytes from the HSV-2 seronegative patient, suggesting that HSV-2 does not alter the IFN-γ signaling pathway in HSV-2 naïve human monocytes. In contrast, HSV-2 interferes with the IFN-γ signaling STAT-1 phosphorylation pathway in HSV-2 seropositive patients. The observed down-regulation of IFN-γR caused by HSV-2 exposure is likely to significantly reduce the intensity of the signal induced by IFN-γ stimulation. This may have impact in the ability of the monocyte to effectively mount an interferon gamma mediated antiviral response.

Figure 17 c, d

STAT1 protein +/- HSV-2 stimulation in Seropositive patient +/- IFN- γ



Similar to the experiment on monocytes from HSV-2 seronegative individuals, monocytes from an HSV-2 seropositive person were exposed to HSV-2 with or without IFN-γ. Then Western blots were probed with anti-phosphoSTAT-1 antibody, and the intensity of bands (Figure 17c) was analyzed by densitometry (Table 6). The densitometric analysis shows that the intensities of STAT-1 phosphorylation were 17.75% in media control and 5.20% in UV-inactivated HSV-2 Ag incubated monocytes, both in the presence of IFN-γ (Table 6). These values correspond to the 84kDa band. However, the 91kDa band disappeared in HSV-2 exposed monocytes from the HSV-2-seropositive person. Intensities of STAT-1 proteins were 8.94% and 7.60% in media control with and without IFN-γ stimulation, respectively. In samples incubated with HSV-2, STAT-1 protein is similar with and without IFN-γ stimulation, with percentage of volume 28.73% and 25.62%, respectively (lanes 3 and 4; Table 6). Results demonstrated that HSV-2-incubated monocytes have significantly greater total STAT1 protein levels. In HSV-2 incubated monocytes, STAT-1 phosphorylation is weaker than in media control samples (Figure 17c). The STAT-1 phosphorylation ratio is 1.98 and 0.18 in media and HSV-2 incubated monocytes, respectively. These results suggest that HSV-2 interferes with the IFN-γ signaling STAT-1 phosphorylation pathway in HSV-2 seropositive patients. In addition, exposure to HSV-2 ablates the 91kDa STAT-1 band. The observed down-regulation of IFN-γR caused by HSV-2 exposure is highly likely to reduce the intensity of the signal induced by IFN-γ stimulation and therefore the antiviral response of monocytes in HSV-2 seropositive people.

However, it does not explain why there was IFN-γR down-regulation when the experiment was performed in whole PBMC. There may be more than one mechanism for the regulation of the IFN-γR in monocytes. It may be that despite the down-regulation the ligand-receptor-signaling pathway remains intact in HSV-2-seronegative patient. This is in contrast with monocytes from HSV-2-seropositive individuals, where there is a defective response to IFN-γ after re-exposure to viral antigen. This deficit could be innate or acquired, but at the moment this is unknown. However, this result requires further study and confirmation. It is still not clear whether HSV-2 can directly interfere with IFN-γR signaling pathway.

To confirm the validity of the results of the STAT-1 experiment, HLA-DR expression on the surface of monocytes was also examined. Since HLA-DR expression is regulated by the IFN-γ pathway, the effects of IFN-γR down-regulation on IFN-γ mediated functions were studied. PBMC from HSV-2-seropositive patients were exposed to UV-inactivated HSV-2 for 24hours, and HLA-DR expression was measured by flow cytometry. Results showed that HLA-DR expression increased upon HSV-2 exposure (Figure 18a), since the IFN-γR expression and its MCF rose from 12.02 to 23.16. Using PBMC from seronegative patients, HLA-DR expression increased from 15.84 to 29.56 after incubation with UV-inactivated HSV-2 Ag (Figure 18b).

Increased expression of HLA-DR was similar in both HSV-2-seropositive and seronegative patients, perhaps because few IFN-γ receptors are required to induce the expression of HLA-DR in response to IFN-γ. However, an alternative explanation to this result is that HLA-DR expression is not only activated by the IFN-γ pathway but also by other pathways. In addition, transcription factors other than interferon associated genes

might be involved in the induction of HLA-DR expression. Furthermore, the sensitivity and responsiveness of HLA-DR to the activation IFN-γ pathway is not well characterized.

Table 6. Densitometry analysis of Western blot films to determine STAT 1 phosphorylation in monocytes from HSV-2 seropositive and seronegative individuals.

Refer to Figure17A and 17B	Seronegative			
	media+IFN	media	HSV-2+IFN	HSV-2
STAT-P	22.5		77.5	
STAT-1	15.94	17.28	36.84	29.94
The relative value of	1.41		2.1	
STAT-1 being phosphorylated				
Refer to Figure 17C and 17D	seropositive			
	media+IFN	media	HSV-2+IFN	HSV-2
STAT-P	17.75		5.2	
STAT-1	8.94	7.6	28.73	25.62
The relative value of	1.98		0.18	
STAT-1 being phosphorylated				
STAT-P represents the intensity of the band of phosphorylated STAT-1				
STAT-1 represents the protein level of STAT-1				

Objective:

To investigate whether HSV-2 induced IFN-γR down-regulation has an impact on the signaling pathway used by IFN-γ (see figure 17 footnote).

Densitometry results from Figures 17a-d, demonstrating the intensity of STAT-1 phosphorylation bands and its corresponding total protein level following HSV-2 and/or HSV-2 exposure in both HSV-2 seronegative and seropositive persons.

Figure 18a:

Seropositive HLA DR expression

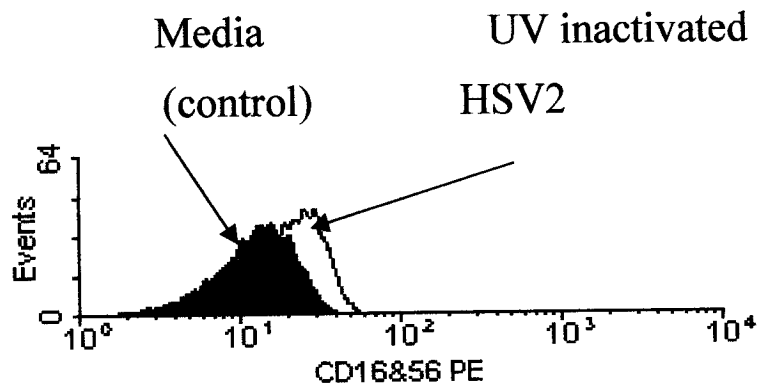


Figure 18b

Seronegative HLA- DR expression

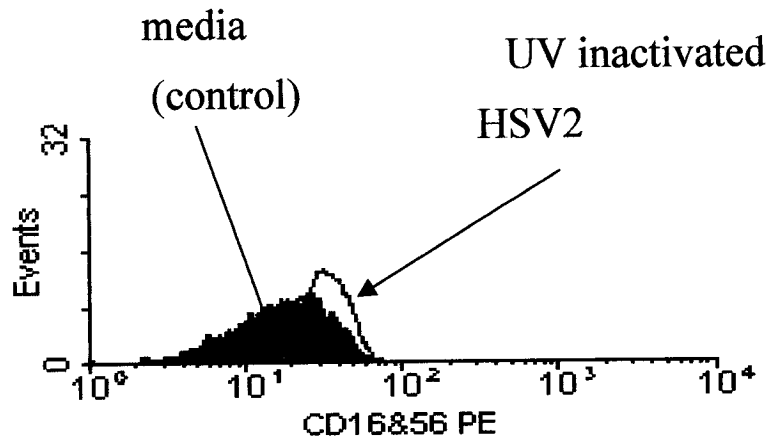


Figure 18a, b:

Objective

HLA-DR expression on the surface of monocytes was also examined, seeking to validate the results of the STAT-1 experiment. Since HLA-DR expression is regulated in part by the IFN-γ pathway, the effects of IFN-γR down-regulation on IFN-γ mediated functions were studied. PBMC from HSV-2-seropositive patients were exposed to inactivated HSV-2 for 24hours, and HLA-DR expression was measured by flow cytometry.

HLA-DR expression in media and UV inactivated HSV-2 Ag incubated PBMC from e) HSV-2 seropositive and HLA-DR expression increased upon HSV-2 exposure, since the IFN-γR MCF rose from 12.02 to 23.16.f) seronegative patients, HLA-DR expression increased from 15.84 to 29.56 upon HSV-2 and PBMC incubation.

4.- Other Potential Mechanisms of IFN-γR Regulation

Thrombin does not cause down-regulation of IFN-γR.

Previous research suggests that thrombin may play a role in HSV-2 infection. According to a study by Pryzdial et al., CMV, HSV-1 and HSV-2 initiate thrombin production (85). To investigate the effects of thrombin production on the down-regulate of the IFN-γR, PBMC were cultured with 27.5 units of thrombin for 24 hrs (Figure 19). Thrombin has been shown to interact with the herpes virus envelope and the cell surface.
Results indicated that thrombin does not cause IFN-γR down-regulation in seropositive patients (Figure 19). However, it is possible that 27.5 units of thrombin may not be sufficient to induce an effect on IFN-γR expression.

Cytokine and Cytokine Receptors, TNF superfamily and chemokine expression change on Human monocytes

To determine whether any other changes of gene expression in monocyte of UV-inactivated HSV-2 cultured PBMC were present by microarray analysis. This analysis would not only provided the change of IFN-γR gene expression but also the change of other cytokine, TNF superfamily and chemokine gene expression. Human PBMCs were incubated in the presence and absence of inactivated HSV-2. After 24 hour incubation, monocytes were isolated from PBMC, and RNA was extracted from the samples. RNA samples were reverse transcribed to produce ^{32}P-labeled cDNA, which was hybridized to filter microarrays containing 375 immune genes. These immune genes include adhesion molecule, angiogenic factor, cell surface protein, chemokine, chemokine receptor, cytokine, binding protein, cytokine receptor, epidermal growth factor, ephrin, ephrin

receptor, fibroblast growth factor, integrin, interleukin, interleukin receptor, neurotrophic factor, protease or related factor, orphan receptor, TGF B superfamily, and TNF superfamily.

Gene expression was compared between inactivated virus exposed samples and control samples cultured in media alone. The difference of mRNA expression is presented in two ways. The first one is the net difference in mRNA intensity between samples treated with UV-inactivated Ag and media only. The second one is the ratio between the intensities. Among all genes analyzed, the TRAIL and chemokine genes have the most significant changes after HSV-2 exposure. In a previous study, 3 folds of change in gene expression has been considered as a cut off to determine which gene has changed in expression before and after treatment (93). In HSV-2-seropositive patients, chemokines such as MCP-1, MCP-2, MCP-3 and IP-10 were significantly up-regulated, the ratios of increased gene expression were greater than 3-fold.

The expression of chemokines was only slightly changed in seronegative patients, the ratio of all the gene expression changes was less than 3-fold (Table7). CD40 mRNA expression increased significantly in HSV-2-seropositive patients from 0 to 11480.8 (units are a measure of intensity; the ratio was greater than 3 fold), but it was not affected in seronegative patients. The significant increase in chemokine and CD40 gene expression suggests that monocytes are differentiating into macrophages after exposure to HSV-2 in seropositive patients.

TRAIL mRNA increased significantly in the HSV-2 incubated monocytes compared to the control media sample in the HSV-2-seropositive patient (ratio was 4.5 fold after HSV-2 Ag exposure). However, in the seronegative monocyte sample, TRAIL

mRNA only increased slightly after HSV-2 incubation (change was less than 3 fold). The significant change in TRAIL mRNA might indicate that the monocyte/macrophage increases their cytotoxicity by mediating apotosis through the increase expression of TRAIL on the cell surface in seropositive patient PBMC.

Table 7 also illustrates that TRAIL R1 mRNA levels increase in seropositive samples. The net difference of mRNA intensity was 2722.58, but the ratio of mRNA change cannot be determined, since the intensity of media control became 0 after background adjustment (it is represented with symbol *a* in table 7). While in seronegative samples, the TRAIL R1 mRNA levels changed very little. Both Fas gene and TRAIL R3 expression showed certain increases as well and their net changes of mRNA intensity were 9654 and 1066, respectively. Once again, their ratio of mRNA change cannot be determined simply because the control value became 0 after background adjustment.

Effect of Thrombin on the IFN-γR down-regulation after 24 hrs incubation

Figure 19:

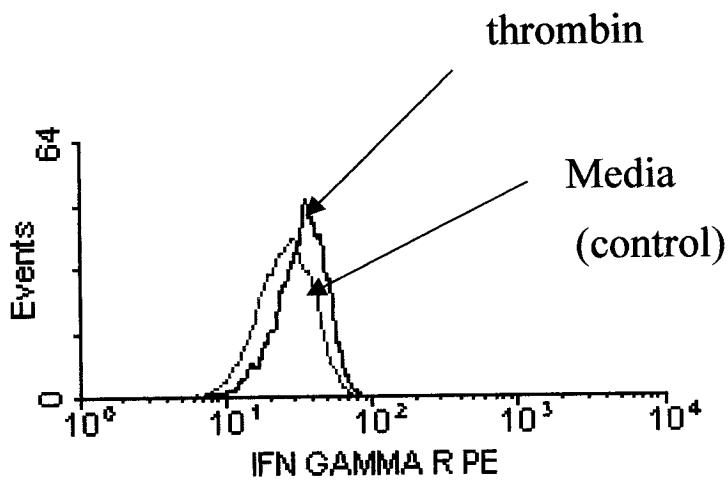


Figure 19:

Objective:

To investigate the effects of thrombin production on the down-regulate of the IFN-γR. Thrombin has been shown to interact with the herpes virus envelope and the cell surface.

PBMC were cultured with 27.5 units of thrombin for 24 hrs (Figure 16). IFN-γR expression in PBMC monocytes is measured as mean channel fluorescence (MCF) following exposure to thrombin.

Table 7 Filter microarray results. Cytokine receptor family, TNF superfamilies, and chemokine family mRNAs in monocytes measured in units of relative intensity following HSV-2 exposure in both HSV-2 seropositive and HSV-2 seronegative patients.

	Seropositive		Seronegative	
Gene Name	HSV2-Control Net changes	Ratio of mRNA HSV2 : control	HSV2-Control Net changes	Ratio of mRNA HSV2 : control
B7-2	-329.59	<3	164.895	<3
B7-1	2234.51	<3	558.065	<3
	Seropositive		Seronegative	
CD40	11480.815	3:1	2.795	<3
TRAIL R1	2722.58	*a*	310.035	<3
TRAIL	33891.7	4.5:1	426.035	<3
TRAIL R3	1066.98	*a*	-292.96	<3
TRAIL R2	395.13	<3	30.855	<3
Fas	9654.28	*a*	-36.595	<3
FasL	0	<3	0	<3
TNF-α	722.94	<3	-22530.18	1:3
	Seropositive		Seronegative	
MCP-1	174659.28	7.5:1	1116.54	<3
MCP-3	42269.21	5.7:1	-983.85	<3
MCP-2	114586.59	11.8:1	510.98	<3
IP-10	87792.845	15.7:1	1150.415	<3

The purpose of this experiment was to examine whether the UV inactivated HSV-2 Ag induced gene expression change of other genes such as chemokine and TNF superfamily genes. Filter cDNA microarrays were used to examine changes in gene expression after UV-inactivated HSV-2 Ag exposure.
Monocytes were purified after PBMCs were incubated with or without HSV-2. RNA was extracted from the purified monocyte. Monocyte RNA was reverse transcribed and labeled followed by hybridization to the microarray filter.

HSV2-control net change represents the difference of mRNA level between monocytes with PBMC cultured with UV-inactivated HSV-2 and media control. Ratio of mRNA of HSV2: control represents the ratio between the mRNA level of monocytes with PBMC culture with UV inactivated HSV-2 and control media.

Symbol *a* represent the ratio cannot be presented because the mRNA level of media control is 0 after background adjustment.

Chlorpromazine inhibits the IFN-γR down-regulation in monocytes

To examine whether clathrin-coated pits were involved in the down-regulation of the IFN-γR, chlorpromazine was used in this experiment. Since chlorpromazine can inhibit clathrin dependent endocytosis, I used chlorpromazine in two different concentrations, 25μM and 50μM. The 25μM of chlorpromazine interfered with IFN-γR down-regulation on monocytes. PBMC from seropositive patients were cultured with UV-inactivated HSV-2 Ag and chlorpromazine for 24 hrs. The negative control was DMSO cultured with PBMC and DMSO plus HSV-2 Ag cultured with PBMC. The reason of using DMSO in negative control was that DMSO was used to dilute chlorpromazine. The IFN-γR expression did not change in the culture with DMSO (Fig. 20). The IFN-γR expression have the same extent of down-regulation in the HSV-2 Ag cultured same with or without DMSO as demonstrated in Fig 20, there was complete inhibition of IFN-γR down-regulation after chlorpromazine incubation.

The effect of Chlorpromazine on IFN-γR expression

Figure 20

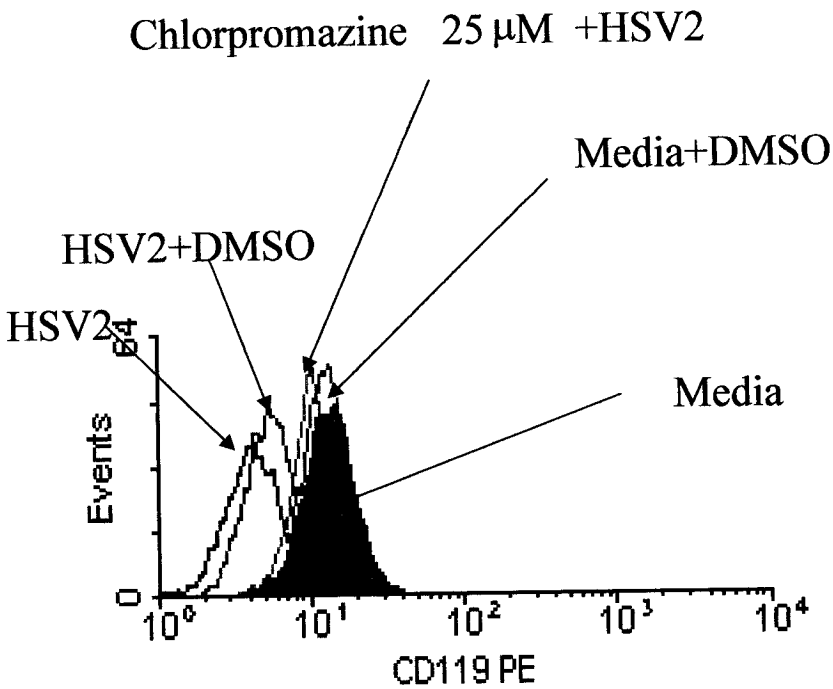


Figure 20.

Objective

To examine whether clathrin-coated pits were involved in the down-regulation of the IFN-γR, chlorpromazine was used in this experiments.

Chlorpromazine was added at two different concentrations, 25μM and 50μM. The 25μM dose of chlorpromazine interfered with the HSV-2 induced IFN-γR down-regulation on monocytes. The negative control was DMSO cultured with PBMC and DMSO plus HSV-2 Ag cultured with PBMC. The IFN-γR expression did not change in the culture with DMSO.

DISCUSSION

Previous observations from this laboratory show that HSV-2 seropositive patients with recurrent genital herpes have defects in IFN-γ mediated anti-viral activity (82). This result prompted the hypothesis that the defect in IFN-γ mediated antiviral effects may be due to a functional deficit in the IFN-γR. Hence, the regulation of IFN-γR by HSV-2 was examined in the present investigation using two *in vitro* cell models and one *ex vivo* experimental model: 1) THP-1 pre-monocytic cell lines, 2) neuroblastoma cell lines, 3) PBMC from HSV-2-seronegative and seropositive patients at different stages of disease. Previous work on THP-1 cells demonstrated that the effects of IFN-γ are mediated through STAT-1 signaling (94). THP-1 cells are a pre-monocytic cell lines and they were assumed to have similar biological properties as PBMC monocytes. Thus, THP-1 was one of our experimental models. Neuroblastoma cells are derived from neuron cells; they provide a good model of HSV-2 neural invasion.

The results of this investigation indicate that UV- inactivated HSV-2 Ag induces down-regulation of IFN-γR only in monocytes of PBMC origin (Figure 1a). THP-1 and neuroblastoma cells show no change in IFN-γR expression in response to HSV-2 exposure (Figures 2, 3). Based on frequency of HSV-2 recurrence, it was observed that seropositive asymptomatic, seropositive recurrent and seronegative patients all have differential IFN-γ levels (82) and possibly also different IFN-γR expression on monocytes. It is also hypothesized that greater down-regulation of IFN-γR corresponds to greater decrease in IFN-γ antiviral effect. As shown in Figure 1a, asymptomatic and recurrent patients exhibit similar levels of IFN-γR down-regulation. The difference between the levels of IFN-γR down-regulation observed in recurrent versus

asymptomatic patients suggests that UV-inactivated HSV-2 Ag affects IFN-γR expression; however, the idea that differential IFN-γR expression on monocytes influences recurrent disease is not supported by the present investigation. Such similar levels of IFN-γR down-regulation in both monocytes from seronegative and seropositive individuals suggest that the IFN-γ pathway is not the only factor governing HSV-2 latency and reactivation.

Various levels of IFN-γ secretion are observed in asymptomatic, recurrent, and seronegative patients (82). Wald *et al.* (2000) demonstrated that viral shedding is similar in both recurrent and asymptomatic patients (20). This suggests that viral shedding may be independent of IFN-γ secretion. However, our group found a correlation between viral shedding and IFN-γ secretion levels (82). Different experimental designs in these studies could explain these differences.

While neuronal viral load varies in the presence and absence of IFN-γ, the virus is still able to reactivate as long as it reaches its threshold quantity (24). Therefore, viral loads may be different in recurrent and asymptomatic patients, and these differences may correspond to functional differences in the IFN-γ pathway. However, there is no evidence to support the correlation between viral load and likelihood of recurrence in humans.

The figure 1 represents one of the results of several experiments done. Recurrent and asymptomatic patients show greater IFN-γR down-regulation compared to seronegative patients (Figures 1a, b, c), suggesting a correlation between intensity of down-regulation and previous exposure to HSV antigens. IFN-γR down-regulation is only observed in monocytes, not in cell lines. Since the mechanism of IFN-γR down-regulation on the surface of monocytes is not well understood, it is further investigated

here. Based on our results, IFN-γR down-regulation occurs as early as 4 hours after exposure to HSV-2 Ag. Similar results by Hussain *et al.* (1999) show that *Mycobacterium avium* (*M. avium*) infection requires 4 hours to reduce IFN-γRα chain protein expression. Thus, IFN-γR down-regulation may require at least 4 hours after exposure to microbial elements.

Glycoprotein gD2 is crucial in viral fusion and attachment to host cell surfaces; its effect on IFN-γR expression has been observed before (126). Asymptomatic and recurrent patients show similar IFN-γR down-regulation in response to gD2 exposure. Our hypothesis is based on a correlation between antiviral effects and IFN-γR expression; observation of IFN-γR down-regulation by gD2 suggests that these viral proteins induce the same inhibiting IFN-γR effect in both asymptomatic and recurrent patients. However, this effect is most likely fully mediated by IFN-γ binding to its receptor, followed by internalization of the ligand/receptor complex. The IFN-γ antiviral effects may also correlate with frequency of reactivation. However, studies in a murine model show that IFN-γ can suppress HSV infection but cannot control reactivation (24). Therefore, IFN-γR down-regulation observed in asymptomatic and recurrent patients may not affect HSV-2 reactivation from neuronal latent infections, but it may influence HSV-2 replication and infection of epithelial cells, once the virus is released from neuronal endings.

Secretion of IFN-γ in response to HSV-2 Ag is higher in asymptomatic patients than recurrent and negative patients (82). As discussed above, IFN-γ secretion may cause down-regulation of IFN-γR through binding followed by internalization. In addressing

this, it appears that neutralizing antibodies only partially inhibit down-regulation in both asymptomatic and recurrent patients. It follows that HSV-2 induced down-regulation of IFN-γR is mostly independent of IFN-γ expression. Similar experiments with gD2, which is one of the viral envelope proteins, demonstrated that the whole inactivated virus had different effects on the IFN-γR. The effect of gD2 was IFN-γ dependent as demonstrated by the neutralization experiments. Use of other glycoproteins such as gD1 and gC1 each resulted in IFN-γR down-regulation, meaning that IFN-γR regulation is not specific to gD2. Preliminary results performed but not reported here, demonstrate that influenza virus, cytomegalovirus (CMV), and adenovirus all induce IFN-γR down-regulation, implying that the down-regulation of IFN-γR may be a general antimicrobial mechanism, which is not HSV specific, but may involve different pathogens.

The secretion of soluble factors plays an important role in receptor regulation. For instance, TNF-α and IL-6 increase IFN-γR gene expression by regulating its transcription (95). The initial results demonstrate that TNF-α levels increase after 5 consecutive days of HSV-2 Ag exposure (Not shown). Based on earlier work, this increase in TNF-α should have induced IFN-γR up-regulation instead of the down-regulation observed (96). Similarly, IL-1 is known to cause up-regulation of IFN-γR expression and induce HLA-DR expression on human monocytes and THP-1 cells (96). Therefore, soluble factors are unlikely to contribute to the HSV-2 induced IFN-γR down-regulation. A similar conclusion was suggested from the experiments in the cell lines.

IFN-γR expression on THP-1 and neuroblastoma cells is not affected by the secretion of soluble factors from HSV-2 exposed PBMC. In a previous study, IFN-γR was internalized upon the addition of IFN-γ to THP-1 cells (97), indicating that IFN-γR

expression can be modulated by IFN-γ. Possibly, IFN-γ in the supernatant from seropositive PBMC may be too low to induce the down-regulation of IFN-γR on THP-1 cells. Alternatively, other factor may be influencing the expression of IFN-γR resulting in a neutral expression level.

To further investigate the possibility that secretion of soluble factors induces IFN-γR down-regulation, PBMC and THP-1 cells were separated using a semi-permeable membrane, allowing the passage of soluble factors. Confirming the previous result, THP-1 cells show no IFN-γR down-regulation mediated by soluble factors secreted from HSV-2 exposed PBMC. In contrast, sorted monocytes showed small decreases in IFN-γR expression when separated from HSV-2 exposed PBMC by a semi-permeable membrane. The discrepancy between might be due to the relative sizes of membrane pores and HSV-2 virion size (400nm versus 150nm). HSV-2 Ag might diffuse slowly from the top to the bottom. In normal monocytes contained in PBMC, the net MCF shift of IFN-γR expression upon HSV-2 exposure was 8.54 units. The net MCF shift of IFN-γR expression upon exposure to soluble factors on sorted monocytes was 3.31 units. This small shift caused by soluble factors cannot explain the full extent of IFN-γR down-regulation in seronegative patients. Since the decrease in IFN-γR expression cannot be explained solely by the secretion of soluble factors, the importance of cell-cell interactions was also investigated.

In examining the effects of intercellular interactions, IFN-γR down-regulation was examined in populations of PBMC with single cell types depleted one at a time. The objective of these experiments was to examine the importance of cell-cell interactions in the down-regulation of IFN-γR and to determine which cell populations were responsible

for this regulation. Comparing the extent of changes in IFN-γR expression observed in PBMC with changes observed in the T cell depleted PBMC demonstrated a diminished effect on IFN-γR in the depleted experiment. Depleting T cells reduces the number of cytokines secreted by these cells, and cytokines like IFN-γ can cause partial down-regulation of IFN-γR. T cells may play a major role in the regulation of IFN-γR by both interferon dependent or independent pathways. B cell depleted samples show significant IFN-γR down-regulation, indicating that the depletion of B cells does not affect IFN-γR down-regulation.

In contrast, to the effects of T cell depleted samples, NK cell depleted samples demonstrated synergistic effects with HSV-2, the down-regulation observed was more pronounced than that of whole PBMC, suggesting that NK cells may help maintain a stable expression of the IFN-γR. This was an unexpected result, as NK cells are known to produce most of the IFN-γ after exposure to HSV. Theoretically, in the absence of NK cells and with lower levels of interferon gamma, one should expect less IFN-γR internalization.

Further evidence to establish the involvement of cell-cell interactions in IFN-γR down-regulation was obtained from the study of purified and non-purified monocytes. Similar to monocytes in PBMC, purified monocytes showed a comparable decrease in IFN-γR expression upon exposure to HSV-2 Ag, indicating that the interaction of monocytes with other cell types is not required for IFN-γR regulation by HSV-2 in seropositive patients. The depletion experiments confirmed that the down-regulation of IFN-γR was not mediated exclusively by intercellular interactions. In contrast, cells from a HSV-seronegative volunteer showed down-regulation of the IFN-γR after incubation of

unseparated PBMC but not after isolated monocytes were cultured with HSV. This suggests that monocytes from HSV-2 seronegative patients are different from the HSV-2 seropositive counterparts. Under this circumstance, monocytes may require an innate immune response dependent on cell-cell interaction to regulate IFN-γR expression. The regulation of IFN-γR in HSV-2 seronegative patients is IFN-γ independent which is in contrast to HSV-2 seropositive patients in whom IFN-γ, as part of the acquired immune response against the virus, has influence on IFN-γR regulation.

It is suggested that the regulation of IFN-γR in response to exposure to HSV is different in HSV-seronegative individuals. It is also likely that individual differences exist among individual in the regulation of this molecule.

The interaction of CD28 with B7 is important in the stimulation of T cells by antigen presenting cells (98). Hence, the effects of CD28/B7 interactions on IFN-γR regulation were investigated. In a previous study by Edelmann *et al.* (2001), disrupting interaction between CD28/B7 and CD40/154 abolished anti-HSV CD4+ and CD8+ T cell responses (99). Since it has been demonstrated that the co-stimulatory signal plays a role in host defense against HSV infection (99), the ability of this interaction to affect IFN-γR regulation was observed. The apparent decrease in CD80 and CD86 expression occurs because CTLA-4 competes with the anti-CD80/CD86 antibody used to examine receptor expression. This appears to cause CTLA-4 mediated CD80 and CD86 down-regulation. IL-2 concentration decreases to almost half of the original level following the addition of 1μg/ml CTLA-4, but higher concentrations of CTLA-4 do not additionally decrease IL-2 secretion. This suggests that CD28/B7 interactions cannot be blocked further and do not participate in IFN-γR down-regulation.

Soluble factor secretion and intercellular interactions may explain only in part the likely mechanisms responsible for IFN-γR down-regulation; although, it is possible that *de novo* protein synthesis is also involved in this regulation. CD4 is down regulated upon HIV infection (90); it is also mediated by *Nef* protein generation. The addition of CHX inhibits *Nef* protein expression; therefore, CHX can inhibit CD4 down-regulation. Similarly, CHX was used to inhibit protein synthesis in both monocytes and lymphocytes (such as T, B, and NK cells) in order to examine effects on IFN-γR down-regulation in monocytes. This may prevent the production of cellular proteins that trigger IFN-γR down-regulation. Previous work on CHX demonstrates that after 24 hours of exposure, 10μg/ml of CHX inhibits protein synthesis without toxic effects to PBMC (92). In a study by Durig *et al.* (2000), 2 μg/ml of CHX was used to inhibit protein synthesis without toxic effect (91). The concentrations of CHX used in this study (1 and 2 μg/ml) do not affect IFN-γR down-regulation. Therefore, these results suggest that HSV-2 Ag does not induce the synthesis of new protein(s) involved in IFN-γR down-regulation.

The observations in this thesis confirm results found by Mossman *et al.* (2001), who determined that the IFN-γ stimulated antiviral response to HSV-1 does not require *de novo* protein synthesis but does require viral entry (71). It is not surprising to find similar results with HSV-1 and HSV-2, since HSV-1 and HSV-2 are 50% homologous and share some common biological properties (100). Because UV inactivated HSV-2 Ag is non-infectious, there is no HSV-2 replication, and therefore no new viral proteins are generated in the HSV-2 exposed cells. This did not satisfy with the requirement of viral entry in Mossman study of activating the IFN-γ antiviral response, such as induce interferon-stimulated gene (ISG) expression. Therefore, UV inactivated HSV-2 Ag

should be expected to be less effective in stimulating IFN-γ response in my experiment, such as chemokine production. However, the opposite result was observed in the microarray result, which showed an increase in interferon inducible gene expression. In the case of HCMV, the interferon-stimulated gene (ISG) is STAT independent; though, it is associated with a new transcriptional factor called interferon-stimulated gene factor 3 (ISGF3) (101). ISGF3 binds to a general enhancer element, which is known as the interferon-stimulated response element (ISRE), which may be an alternative element, besides STAT-1, in the antiviral response to HSV.

Based on Western blot results, HSV-2 exposed monocytes in the HSV-2-seropositive person undergoes no phosphorylation of the 91 kDa isoform and less phosphorylation of the 84 kDa STAT-1 protein. Hence, HSV-2 interferes with the IFN-γ JAK/STAT pathway. This observation is novel and to my knowledge it has not been reported before. However, these results will require confirmation in more than the two HSV-2 seropositive patients we examined for signal transduction function.

Previous research shows that IFN-γ can induce the IP-10 in monocyte (102). In the microarray result of seropositive volunteer sample, IFN-γ inducible gene expression such as IFN-inducible protein (IP-10) is induced upon exposure to UV- inactivated HSV-2 Ag. Despite the down-regulation of IFN-γR and interference in the IFN-γ pathway, the IP-10 expression was still up-regulated. These results imply that ISG induction may not be completely mediated through the IFN-γ pathway. It has been suggested that IFN-α can also induce ISG such as IP-10 (103). However, Hussain *et al.* (1999) demonstrate that *M. avium* infection inhibits the induction of IFN-γ-inducible genes in mouse macrophages by down-regulating IFN-γR (104), though IFN-γ stimulated gene pathways may be different

in humans and mice. In 2001, Mossman *et al.* proposed that viral entry is required for ISG induction, and that this induction may not be mediated through the IFN-γ pathway (71). This implies that inhibition of ISG induction may require viral entry in my experiment.

In recent studies, nectin was shown to be a mediator of alpha herpes virus entry (12;105). Further investigations are required to determine the binding and endocytotic cycle of HSV-2 and whether specific viral products are involved with the change in cell membrane molecules.

Monocyte chemoattractant protein (MCP) belongs to the CC chemokine family and is an important factor in innate immunity (106). MCP is also important in the development of Th2 immune responses (107). Several cell types, such as T cells, secrete MCP-1 in response to LPS and cytokines such as IL-1, IL-4, IFN-γ, and TNF-α (108). Gene expression of IP-10 and MCP-1, -2, and -3 are significantly up-regulated in monocytes of HSV-2-seropositive patients (Table 7). Compared to monocytes from seropositive patients, monocytes from seronegatives show a smaller increase in chemokine gene expression. Previous work by Krakauer and Oppenheim (1993) demonstrates that expression of the same chemokine genes is significantly increased in IFN-γ stimulated macrophages (52). This gene expression is mediated by increased transcription, and it is correlated with binding at IFN stimulus response element (ISRE) and κB sites (52). These sites are not involved in signaling through the JAK/STAT pathway. In addition to chemokine genes, the microarray experiments show that CD40 gene expression is significantly up-regulated in monocytes from HSV-2 seropositive patients, though not in seronegative. Increased expression of chemokine and CD40 genes

implies that monocytes are differentiating into macrophages in seropositive samples. This differentiation may be the key to the mechanism of IFN-γ down-regulation.

Since IFN-γR down-regulation is not mediated by *de novo* protein synthesis, the regulation of this receptor may occur at the mRNA level. Two studies demonstrate that adenovirus E1A and human papilloma virus E6 and E7 proteins inhibit production of IFN at the transcriptional level (109;110). Furthermore, IFN-γRα chain mRNA levels in seropositive patients are unaffected by HSV-2 exposure, while levels increase slightly in seronegative patients. This suggests that IFN-γR down-regulation does not occur at the mRNA level. IFN-γRβ chain mRNA levels increased in HSV-2 seropositive patients in comparison to seronegative patients. The increase in IFN-γRβ chain mRNA in seropositive patients might be a compensation for IFN-γR down-regulation. Hussain *et al.* (1999) show that *M. avium* infection causes IFN-γR down-regulation by a mechanism that involves a decrease in the expression of IFN-γR chain 1 and 2 mRNA (104). In contrast, IFN-γR mRNA expression is unaffected by HSV-2.

To investigate the possibility that IFN-γR down-regulation may result in a signaling defect, Western blot analysis was performed. Since phosphorylation changes in the cytoplasmic domain of IFN-γR, JAK1, and JAK2 can affect the phosphorylation of STAT-1, phosphorylation of the downstream, IFN-γ signaling molecule STAT-1 was examined. Results indicate that IFN-γ can induce STAT-1 phosphorylation in the presence of HSV-2 in monocytes from HSV-2 seronegative individuals. Phosphorylation of STAT-1 is more significant after HSV-2 incubation than in control samples. Also, total STAT-1 protein levels are increased by HSV-2. These results suggest that the monocytic

IFN-γ pathway functions properly in HSV-2 seronegative monocytes even after HSV-2 exposure.

The results suggest that monocytes from HSV-2 seropositive patients undergo less STAT-1 phosphorylation after HSV-2 exposure. As noted with HSV-2 seronegative monocytes, STAT-1 protein levels increase significantly in HSV-2 exposed monocytes. However, STAT-1 phosphorylation was decreased significantly by HSV-2 in these monocytes. This suggests that the IFN-γ pathway is defective after HSV-2 exposure in HSV-2 seropositive patients. Hussain *et al.* (1999) found that *M. avium* also blocks STAT-1 activation and tyrosine phosphorylation (104), resulting in reduced IFN-γRα phosphorylation. In addition, inhibition of STAT-1 phosphorylation appears after 8 hours of exposure. Our results show that the extent of STAT-1 phosphorylation is greater in seronegative than seropositive patients. Various levels of IFN-γR down-regulation might differentially affect the integrity of the signaling pathway, suggesting that a threshold number of IFN-γR must be maintained in order to sustain the phosphorylation pathway. However, this threshold is not known.

HLA-DR is a human molecule that is similar to the murine MHC class II molecule (111); its expression can be up-regulated through the IFN-γ pathway (112). One would not expect up regulation of HLA-DR if the IFN-γR pathway was defective in monocytes of seropositive patients. The Western blot results did not confirm this assumption. However, IFN-γ may not be the only factor regulating HLA-DR expression. For instance, IL-1 induces the expression of HLA-DR on human monocytes and THP-1 cells (96). Hussain *et al.* (1999) demonstrated that IFN-γ induced signal transduction is reduced in *M. avium* exposed macrophages, and suppression of the IFN-γ pathway is time

dependent (104), occurring after 8 hours of exposure. Our study measures HLA-DR expression at 24 hours; at this point, an increase in HLA-DR expression is observed, indicating that an unknown alternative pathway might compensate for the suppression of HLA-DR.

To investigate the possibility of competition between HSV-2 Ag and the IFN-γR flow cytometry antibodies, a competition assay was performed. If HSV-2 Ag competes with the staining antibodies on the fixed cell membrane surface, then increasing doses of HSV-2 Ag would decrease the binding of labeling antibodies, and a lower IFN-γR level would be detected by flow cytometry. Increasing concentrations of HSV-2 Ag does not significantly change the binding of labeled antibodies to IFN-γR, indicating that there is no competition between HSV-2 Ag and the IFN-γR antibodies.

The cell depletion experiments demonstrated that NK and T cells may play a role in the IFN-γR regulation. During HSV-2 infection, CD4 T, CD8 T, and NK cells all participate in immune defense. In particular, defects in NK cell cytotoxicity result in increased susceptibility to HSV-2 infection (113). The cells responsible for IFN-γ synthesis and secretion are NK and T cells; studies show that IFN-γ mRNA synthesis increases in PBMC exposed to UV inactivated HSV-2 (114). Other work illustrates that IFN-γ secretion by NK cells is interleukin specific, and only NK cells stimulated with IL-12 or IL-18 can secrete large amounts of IFN-γ(33). It is likely that NK may be required to maintain stability of IFN-γR expression. However, the mechanism by which this happens remains unknown.

According to Durig *et al.* (2000), HSV-1 mediates the *in vitro* enhancement of NK cell activity through the induction of IL-15 (28). The present investigation shows that

IL-15 mRNA levels increase significantly in seropositive patient samples following exposure to HSV-2 Ag. In contrast, IL-15 mRNA levels in seronegative patients decrease slightly in HSV-2 exposed samples. IL-15 derived T and NK cells are not able to produce cytokines (33). The increased IL-15 mRNA observed in seropositive samples suggests an increase in the numbers of T and NK cells that are unable to produce IFN-γ. These variations in NK cell production may be the reason for differential secretion of IFN-γ among asymptomatic and recurrent patients.

Previous work in our lab demonstrates that TNF-α increases upon HSV-2 exposure. There may also be an increase in monocyte TRAIL. With its death domain, the TRAIL receptor (TRAIL R1) can trigger apoptosis (115), which in turn weakens immune response to HSV-2. Table 7 illustrates that TRAIL R1 mRNA levels increase significantly in seropositive samples; in seronegative samples, these levels change very little. The increase was seen in HSV-2 seropositive monocytes and suggests the differentiation of monocytes into macrophages, which could change cell susceptibility to apoptosis. Both Fas gene and TRAIL R3 expression show significant increases as well. Greater TRAIL R3 expression can prevent apoptosis triggered by other cell types; however, the net increase in TRAIL R1 is twice that of TRAIL R3. In all, there is a net increase in the rate of apoptosis. Thus, seropositive monocytes are more easily changed into macrophages and become more susceptible to cell death.

We have shown that HSV-2 induced IFN-γR down-regulation is not mediated by the secretion of soluble factors, *de novo* protein synthesis, or interruption of IFN-γRα chain transcription. We have shown that intercellular interactions involving cytokine-receptor binding, such as T-cells and NK cells may play a partial role in the regulation of

the IFN-γR. We have also demonstrated that the IFN-γ signal pathway does not function properly in the presence of HSV-2 in monocytes from seropositive patients. As demonstrated recently by Sadir *et al.* (2001), IFN-γR is internalized by two different pathways involving clathrin-coated pits and caveolae (116). Adding chloropromazine, which inhibits internalization by blocking clathrin-coated pits, inhibits down-regulation of IFN-γR in response to HSV-2 (Figure 20). This suggests that IFN-γR regulation by HSV-2 involves internalization in clathrin coated pits forming vesicles that trap receptors inside the cell membrane.

In recent studies, nectin has mediated cellular entry of alpha herpes virus (12;105); this raises the question of where HSV-2 Ag binds in the cell membrane. It has been shown that intercellular adhesion mediates tyrosine phosphorylation of nectin-2δ (117). Phosphorylation of the cytoplasmic region of nectin-2δ may be due to the activation of Src kinase (117). As well, SHP-2 and Src kinase are involved in the negative feedback loop of IFN-stimulated JAK/STAT signaling (118). Therefore, the decrease in the portion of STAT-1 phosphorylation might be correlated with the activation of Src kinase. Finding the connection between nectin binding, Src kinase activation and the JAK/STAT pathway is the goal of future work.

The conceptual models that summarize the results in this thesis are represented in Figure 21a for HSV-2 seropositive and 21b for HSV-2 seronegative individuals. The immune mechanisms that HSV-2 seropositive and seronegative individuals share in the regulation of the IFN-γR, include:

- Humoral factors such as IFN-γ and IL-10 do not induce significant down-regulation of IFN-γR in monocyte.

- The IFN-γR down-regulation is not mediated through the change of IFN-γR mRNA level.
- The IFN-γR down-regulation is not mediated by protein synthesis (De novo synthesis)
- The IFN-γR down-regulation is mediated by endocytosis involving clathrin coated vesicles

The mechanism of IFN-γR regulation observed in HSV-2 seropositive patients only, include:

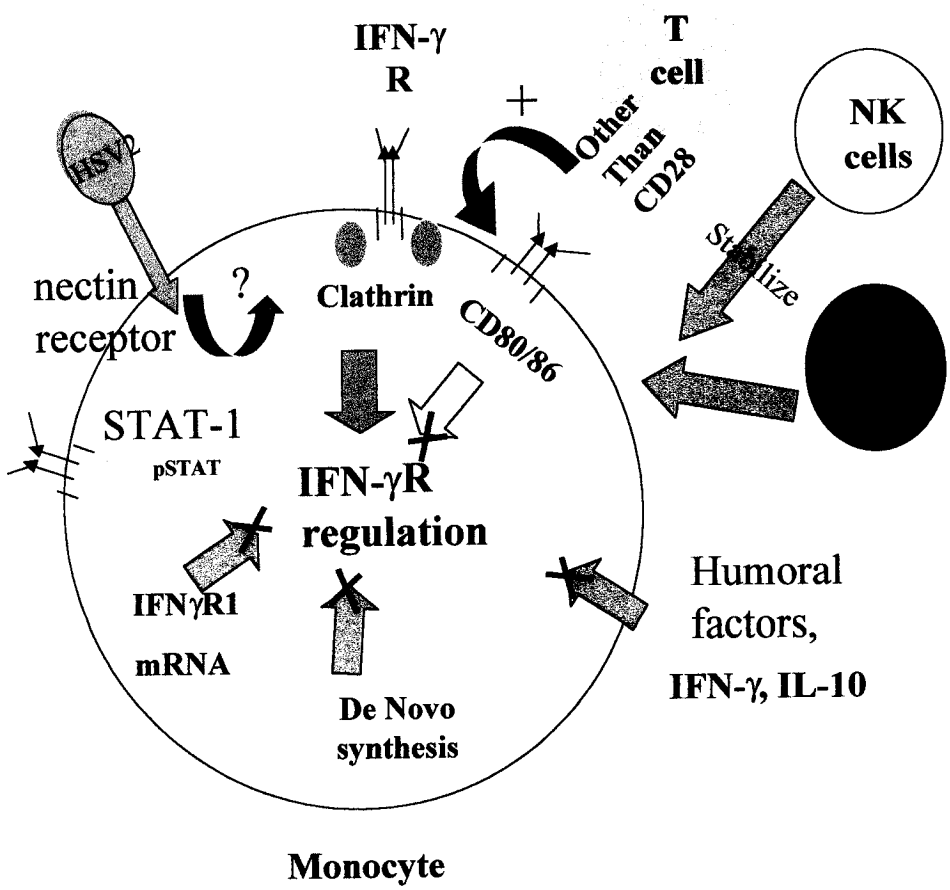
- Cell-cell interaction contributes less to the regulation of IFN-γR on monocyte than in seronegative individuals. T cells may facilitate HSV-2 induced IFN-γR down-regulation, while NK cells may stabilize IFN-γR expression.
- HSV-2 may interfere with phosphorylation of STAT-1 and therefore may impact on the IFN-γ antiviral mechanism in monocytes

The mechanism of IFN-γR regulation observed in HSV-2 seronegative individuals only, include:

- Cell-cell interaction contributes to the regulation of IFN-γR on monocyte. The role of NK cells is not known. T cells may facilitate more the HSV-2 induced IFN-γR down-regulation than in seropositive patients.
- HSV-2 does not interfere with IFN-γR signaling pathway in HSV-2 seronegative patients.

Figure 21a

Mechanisms of Interferon gamma receptor regulation by HSV-2 in Seropositive Patients

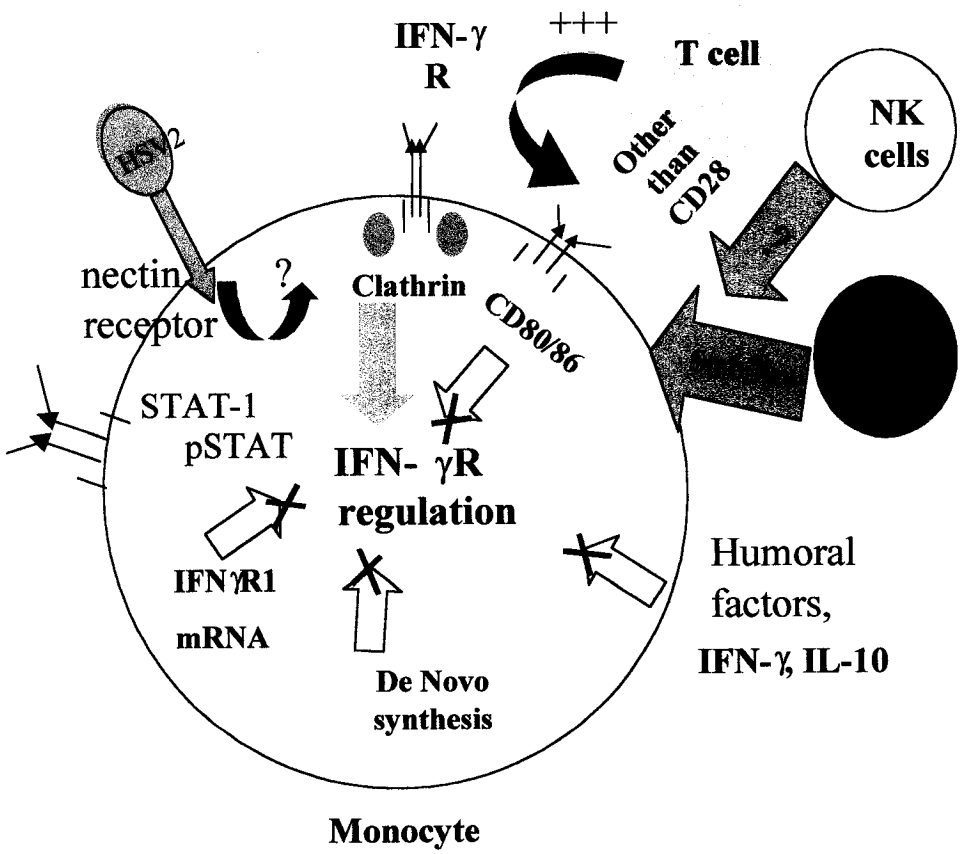


NB: + represent only mininal effect on IFN-γR down-regulation by T cells

Figure 21a. Mechanisms of interferon gamma receptor regulation by HSV-2 in seropositive patients

Figure 21b

Mechanisms of Interferon gamma receptor regulation by HSV-2 in Seronegative Patients



NB: +++ represent strong effect on IFN-γR down-regulation by T cells

Figure 21b: **Mechanisms of interferon gamma receptor regulation by HSV-2 in seronegative patients.**

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